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der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität
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Establishment and validation of a viable *in vitro* skin model
from different species for the evaluation of veterinary and
human cutaneous diseases

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ABBREVIATIONS

AIC	Air interface coculture
API	Active pharmacological ingredient
ASIS	Allergen-specific IgE serology
BBB	Blood-brain-barrier
BCS	Biopharmaceutics Classification System
BP	British Pharmacopeia
BUS	Perfused Bovine Udder Skin-model
CAD	Canine atopic dermatitis
CAFR	Cutaneous adverse food reaction
CL	Cutaneous leishmaniasis
CNS	Central nervous system
CRH	Corticotropin releasing hormone
CYP	Cytochrome P450 enzyme family
Da	Dalton; unit for molecular weight
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
ECVAM	European Centre for the Validation of Alternative Methods
e.g.	Example given
EGF	Epidermal growth factor
etc.	Et cetera
FAD	Flea allergic dermatitis
FDA	Food and Drug Administration (USA)
FDM	Fibroblast-derived matrix model
Fig.	figure
FT	Full-thickness
FTM	Full-thickness collagen

	model
FTO	Full-thickness outgrowth model
GFR	Glomerular filtration rate
H.E.	Hematoxylin-eosin
HPLC	High pressure liquid chromatography
HSE	Human skin equivalent
hsE	Heat separated epidermis
HUVEC	Human umbilical vein endothelial cells
i.e.	Id est—that means/is to say
i.v.	Intravenous
IDT	Intradermal testing
IgE	Immune globuline E
IgG	Immune globuline G
IL-12	Interleukin 12
IL-1 α	Interleukin 1 α
IL-1 β	Interleukin 1 β
IL-8	Interleukin 8
KRB	Krebs-Ringer-Buffer
LADME	Liberation-Absorption-Distribution-Metabolism-Elimination/Excretion
LD50	Lethal dose for 50%
LEM	Leiden epidermal model
logP	Partition coefficient
MC-1R	Melanocortin-1 receptor
MCM	Membrane coating material
MHC-1	Major histocompatibility complex
ML	Mucocutaneous leishmaniasis
MPS	Mononuclear phagocytosis system
MSC	Mesenchymal stem cell
Na ₂ PO ₄	Disodium hydrogen

	phosphate
NaOH	Sodium hydroxide
NFNFDH	Non-flea non-food hypersensitivity dermatitis
NLCs	Nanostructure lipid carriers
NP	nanoparticle
NSAIDs	Non-steroidal anti-inflammatory drugs
OAT	Organic anion transporter
OCT	Organic cation transporter
OECD	Organization for Economic Cooperation and Development
PAMPA	Parallel artificial membrane permeability assay
Papp	Apparent permeation coefficient
PBS	Phosphate buffered saline
PDMS	Poly(dimethylsiloxane)
PUFAs	Polyunsaturated fatty acids
PEG	Poly(ethylene glycol)
pKa	Acid dissociation constant
PGA	Poly-glycolic acid
PLA	Polylactid acid
RHE	Reconstructed human epidermis
ROS	Reactive oxygen species
SC	Stratum corneum
SLNs	Solid lipid nanoparticles
SP	Substance P
TDD	Transdermal drug delivery
T-cell	Thymus-Lymphocytes
TGF- α	Transforming growth factor
TI	Therapeutic index
TNF- α	Tumor necrosis factor
TiO ₂	Titanium dioxide
TLR-2	Toll-like receptor 2
USP	United States

	Pharmacopeia
UV	Ultra violet
VL	Visceral leishmaniosis
ZnO	Zinc oxide

I. INTRODUCTION

Transdermal drug delivery (TDD) is one of the earliest forms of medication employed by humans. For a long time, however, TDD was limited to topical application of e.g. creams and ointments [1]. This is due to the effectiveness of the natural skin barrier which made systemical drug administration via the skin very difficult. With modern technique and pharmaceutical knowledge, this obstacle was passed and the numerous advantages of TDD could be made use of.

Some of these advantages are the following:

- Circumvention of the so-called “first pass effect” by direct absorption of the drug into the circulation. This results in enhanced efficacy of the treatment.
- For TDD generally lower dosages are required than for oral or parenteral drug administration, resulting in decreased risk for toxicity and side-effects. This, together with the aforementioned point, can also help keeping the costs low [1].
- By circumvention of gastrointestinal metabolism, the drug intake can be better controlled and monitored than in most other application ways [1, 2], as the extent and speed of these metabolic processes differ between species and individuals, making predictions about drug behavior often difficult [3, 4].
- This also leads to a higher stability of plasma levels compared to the other methods which may be vital for certain patients [1, 2]
- Application of the drug is easy and pain-free and therefore may increase compliance by the patients and, in case of an animal patient, their owners.

In our current society, an increasing number of people keep one or several pets at home. These animals are ubiquitously seen as a family member and quite often even replace having a child. They tend sometimes to also have the same types of diseases than humans do, and skin related problems are quite common [5-7]. Chronic skin diseases and associated infections with multiresistant organisms are especially difficult to treat. The increasing prevalence of antibiotic resistance has made the treatment much more challenging [8, 9].

Two types of drug administration can be differentiated: Systemic administration and local (topical) administration. Systemic administration by transdermal drug delivery means that the drug permeates through all the skin layers and is then absorbed into the circulatory system and haematogenously transported to the target site. A local effect is achieved when the goal is treating the skin itself, with no need to reach relevant serum concentrations. The ability of any drug to penetrate the skin or permeate through it depends on the galenic formulation. Every drug or active pharmacological ingredient (API) needs a vehicle to transport it to its place of action; sometimes additional agents are required as well. Potential interactions and incompatibilities between the different substances have to be avoided. The pharmacokinetic properties of new drug candidates have to be recognized as early as possible during the development process. When developing a formulation with a new (unknown/uncharacterized) API, frequently only small amounts of the drug will be available because of the high production cost. Therefore, strategies to reduce the costs and shorten and facilitate the preclinical development process are desirable.

Furthermore, ethical concern arose with the gradual change of many animals from a mere resource to companions and friends and the increased criticism of studies using animals for research. The general acceptance of animal experimentation in science decreased considerably during the last few decades [10, 11]. Consequently, new rules and guidelines about animal handling were formulated. In 1956, the 3R-principle was established by William Russell and Rex Burch in their “Principles of humane experimentation techniques” which is still valid today. These three “Rs” stand for *Replacement* of animal testing with another method wherever possible, *Reduction* of animals used if unavoidable and *Refinement* of the experimental conditions in such a way that suffering and pain of those animals are decreased to the absolute minimum [12]. Legislation followed with the creation of the European Centre for the Validation of Alternative Methods (ECVAM) in 1991, the adoption of the “Declaration of Bologna” in 1999, the enactment of Registration, Evaluation and Authorization of Chemicals (REACH) by the European commission in 2007 and further laws in the European and American economic and scientific sectors.

Therefore, transferring the pharmaceutical development from *in vivo* to *in vitro* studies has been the focus of a lot of research in recent years. For such *in vitro* studies appropriate models are required [13]. Although a variety of skin models

for many different purposes exist, most of them can be characterized as “stand alone” approaches rather than validated and standardized procedures that are widely known and accepted [14, 15].

In addition, only few studies concentrate on veterinary drug development. Whether human or animal skin was used, the focus was on human health, and animal models were evaluated solely based on their ability to imitate human skin [14-16].

OBJECTIVE

The overall aim of this dissertation was to develop, validate and establish an *in vitro* skin model with *viable* human and animal skin. In this way it could be of greatest use for investigating skin properties and later disease development. This model ideally should keep the experimental conditions as simple, inexpensive and consistent as possible to enable several laboratories to reproduce the same results and use this model for a wide variety of research studies.

Individual goals of the research were:

- Selection of a suitable marker to determine skin viability and investigation of suitable storage and measurement conditions for this marker in a stability trial
- Adaption and optimization of the viability assay and validation based on FDA bioanalytical guidelines.
- Establishment of a viable human skin model with healthy skin in unsupplemented cultivation medium (DMEM).
- Establishment of a similar model using porcine and canine skin.
- Maintaining the skin samples viable for two weeks in supplemented and basic medium and simultaneous evaluation of the influence of different medium supplements on the skin viability.

II. LITERATURE REVIEW

1. Pharmacology

Pharmacology can be defined as the science of investigating the interactions of substances and organisms in health or disease state, without regard to usefulness or adverse character of these interactions. In a second step by narrowing the definition, pharmacology investigates the interactions between drugs and the organism of human and animal [17]. In a further step, the possible unwanted effects of a substance or drug are analyzed in the field of toxicology. It is of absolute necessity to know about the normal processes in the organism, which is the related field of physiology, to notice if structures and processes in the organism are altered which is the equally related field of pathology [17].

Pharmacology itself can be viewed from two directions:

“How does a substance influence the organism?” This question is addressed by the field of **pharmacodynamics** whereas the question: “How does the organism influence the substance?” may be answered by the field of **pharmacokinetics** [18].

Dose-effect relationship

The potency of a drug is described as the dose or concentration which elicits a defined pharmacological effect. A measure for the potency is the ED_{50} value (effective dose; causing the desired effect in 50% of the tested population, respectively as relative value causing 50% of the possible effect). A drug is the more potent the less the needed concentration for causing an effect. The efficacy of a drug on the other hand measures the extent of an effect caused by a given concentration. The LD_{50} (median lethal dose) value gives information about the dose required to kill half of the tested population. The smaller this number is, the higher is the danger of a lethal effect of a drug. Referring to this value, also a TD_{50} is defined, which indicates toxic effects in 50% of the tested population. The so-called therapeutic index (TI, also known as therapeutic ratio) is a measure to determine the safety of a drug by comparing the dose causing effect with the dose causing toxicity or death [48]:

$$TI = \frac{LD_{50}}{ED_{50}} \text{ or } TI = \frac{TD_{50}}{ED_{50}} \quad \text{Eq. 1}$$

A higher therapeutic index hereby suggests an increased drug safety as the dose needed for therapeutic effects is significantly lower than the dose for toxicity. Based on this index, the therapeutic window can be calculated. It is defined as the range of doses which display an acceptable to optimum relationship between therapeutic and toxic effects. The quotient $\frac{LD5}{ED95}$ is utilized as measure for the therapeutic window. Substances with a narrow therapeutic range have to be applied and monitored with care [19].

1.1. Drug properties

In order to achieve optimum therapeutic efficacy, any drug or drug candidate has to fulfill certain criteria regarding drug potency, target selectivity and the ability to reach a sufficient target tissue concentration. The so-called (L)ADME processes (see the following section 1.2) are of highest importance for defining the disposition of a drug candidate [20]. The optimization of the chemical drug structure in view of the (L)ADME processes is essential for drug discovery and drug design [20]. Significant drug related (L)ADME characteristics are:

- Solubility
- Partition coefficient LogP
- Acid dissociation constant pKa
- Molecular weight
- Protein binding
- Half life

Solubility:

The solubility of a chemical substance, termed as solute, is its ability to dissolve in a solvent by forming a homogenous solution. It is fundamentally dependent on the solvent, on temperature and pressure. The solubility of a substance in a defined solvent is limited by the saturation concentration. Above this threshold concentration, adding more solute to the solution does not increase its concentration further [21]. Solubility can be given as concentration, molality, molar ratio and other units but is for simplicity reasons usually stated as concentration of solute in solvent [21]. Classification into high and moderate (>60µm/mL and 10-60µg/mL respectively) solubility provides a categorization of different drugs [20]. USP (US Pharmacopeia) and BP (British Pharmacopeia) classify solubility regardless of solvent only based on following criteria:

Description	Parts of solvent per part of solute
Very soluble	< 1
Freely soluble	1-10
Soluble	10-30
Sparingly soluble	30-100
Slightly soluble	100-1000
Very slightly soluble	1000-10000
Practically insoluble	≥ 10000

Tab. 1: Solubility classification according USP and BP

LogP:

The water-octanol partition coefficient logP measures the differential solubility of a substance in a hydrophilic solvent (water) and a hydrophobic solvent (octanol). The logarithm of those two values is used for ranking substances according their hydrophilicity/hydrophobicity [21] with higher logP values indicating rising hydrophobicity and resulting better membrane permeating properties [22].

pK_a:

The acid/base dissociation constant, or more exactly its negative decadic logarithm pK_a, is a physicochemical key parameter with an enormous impact on the biopharmaceutical properties of a drug. It defines the tendency of a drug to be present in dissociated (ionized, polar) or undissociated form at a basic or acidic pH. With respect to the fact that most drugs are either weak acids or weak bases [23] it is crucial to know or at least estimate which ionic form a molecule will take at different pH values. A pH matching the pK_a of a drug means that the drug in this condition is present equally in dissociated and undissociated form [22]. In physiological systems the ionization state of a drug influences its ability to diffuse through membranes and overcome the various biological barriers inside the body [23]. The ionized form makes the drug more water soluble (=hydrophilic) while the unionized form provides more lipophilicity, thus enables the drug to pass the nonpolar membranes more easily. Acidic drugs with a pK_a < 7 are present in ionized form in basic environments (pH > 7, such as e.g. blood, small intestine). On the other hand, basic drugs with a pK_a > 7 are ionized in acidic environments (pH < 7, e.g. stomach). Sometimes a basic drug, e.g. morphine with a pK_a of 7.9

which is just partially dissociated in plasma (pH 7.4) arrives in an acidic environment like the stomach (pH around 2), becomes highly ionized there and furthermore cannot diffuse through the gastric lining, unable to leave the stomach. This can have clinical consequences and is referred to as ion-trapping [22]

With the Henderson-Hasselbalch equation the extent of ionization of a drug at a certain pH can be calculated, where A⁻ is the ionized drug and HA the unionized drug [24].

$$\text{pH} = \text{pK}_a + \log_{10} \frac{[\text{A}^-]}{[\text{HA}]} \quad \text{Eq. 2}$$

Molecular weight:

Among other criteria, the molecular weight of a drug determines its absorption and permeation properties. Small molecules, i.e. molecules with low molecular weight, are more likely to diffuse passively through cell membranes than bigger ones. A molecule is considered as small until reaching a mass of around 500 Dalton (Da) to 1000 Da [25]. In the so-called “rule of five”, set by Lipinsky et al. for the determination of “drug likeness” of pharmaceutical drug candidates, the preferable molecular mass is set to 500 Da or less. According to them, a drug candidate should fulfill at least three of the following criteria:

- $\text{LogP} < 5$
- $\text{Molecular weight} < 500 \text{ Da}$
- $\text{Hydrogen donor groups} < 5$
- $\text{Hydrogen acceptor groups} < 10$

It should be mentioned, that adherence to those criteria does not definitely make a molecule drug-like; on the other hand, a non-adherence is not necessarily a disqualification [26].

Protein binding:

While a high lipophilicity, i.e. LogP, increases the ability of a drug to overcome biological barriers, it also increases the drug's tendency to bind to different proteins, especially to albumin and glycoproteins. This protein bound fraction of the drug is hence unavailable. Protein binding of drugs also leads to an elevated risk of toxicity as for clearance the drug needs to dissociate from the protein [20].

Half-life

The terminal half-life $t_{1/2}$ of a drug is the time needed to reduce the drug concentration to 50 % of the initial concentration. Within different compartments (s. drug distribution) a drug may display a different half-life depending on the elimination. The terminal half-life however is defined as the time until half of the drug has left the body. It can be viewed in terms of both clearance and distribution volume (s. there). A long half-life might be due to a low clearance or a high distribution volume or both [27, 28].

1.2. The (L)ADME Processes

LADME is an easy-to-memorize acronym for the processes a drug undergoes between being administered and finally excreted. It stands for the following:

- L Liberation (only applicable for certain dosage forms/formulation)
- A Absorption
- D Distribution
- M Metabolism
- E Excretion

1.2.1. Liberation

The first step liberation describes the release of a drug out of its dosage form and is applicable for all solid, semisolid and liquid dosage forms except the liquids in which the API is already available in molecular disperse solution [29].

1.2.2. Absorption

Absorption defines the process of drug entry from the site of application into the systemic circulation via different application routes [30]. Influencing factors are the physicochemical properties of the drug, administration route, blood flow to the absorption site, available surface area for absorption, contact time to mentioned surface and affinity of the drug to certain tissues [31-33]. To which extent a drug is absorbed depends largely on its bioavailability [34] which is derived from administration route and drug properties.

Determining bioavailability

The bioavailability of a substance defines the fraction of a dose reaching the general circulatory system unchanged. General circulation is usually defined by the experimental sampling site, mostly a peripheral blood vessel. Following oral administration, the drug is transported down the gastrointestinal tract, where only part of the dose might be absorbed due to chemical degradation, physically inactivation by binding or complexation, microbial biotransformation, etc. From the actually absorbed part at a time point x , some amount can be metabolised during transit through the gastrointestinal epithelia, some other amount may be metabolised by the liver by way of biotransformation or biliary excretion. Further elimination is possible between the hepatic vein and the periphery circulation system. So the bioavailability F' (of an orally applied dose) is composed of the different fractions that are not eliminated on the way to the sampling site [34].

$$F' = F_X F_G F_H F_S \quad \text{Eq. 3}$$

F_X is the fraction absorbed, F_G is the fraction that “survives” a single passage through the gastrointestinal epithelium, F_H is the fraction that is not metabolised by the liver in the first passage and F_S finally the fraction that escapes any post hepatic elimination. Therefore, the quantity of non-absorbed and intestinally eliminated drug is $(1-F_X)$.

By Eq. 3 oral bioavailability and its components are formally defined, while exact determination of F_S is rarely needed in practice. The more common definition of oral bioavailability, F , is as following:

$$F = F_X F_G F_H \quad \text{Eq. 4}$$

The main difference to Eq. 3 lies in the definition of “general circulation”. Where F' describes the fraction of an oral dose reaching the sampling site unchanged, F is effectively a means of measure for drug availability to the hepatic venous circulation [34] and ranges between 0 and 1[27].

Clinical assessment of bioavailability is calculated by comparison of integrated i.v. drug concentration to extravascular drug concentration

$$F = \frac{\text{AUC e.v.}}{\text{AUC i.v.}} 100 \% \quad \text{Eq. 5}$$

where F is bioavailability, AUC e.v. is the area under the curve (plasma

concentration) after extravascular administration and AUC i.v. the area under the curve after i.v. application [35].

Kinetics of absorption (elimination)

Drug absorption as well as drug elimination (see chapter 1.2.5) can be described by certain pharmacokinetic models. The rate of absorption/elimination is usually either expressed by a half-life $t_{1/2}$ (required time for absorption/elimination of 50 % of the drug) [36], or by a rate constant k (the fraction absorbed/eliminated per time unit), while k is termed k_a or k_e corresponding to an absorption or elimination process.

The relationship between them is [37]:

$$k = \frac{\ln 2}{t_{1/2}} \text{ or } t_{1/2} = \frac{\ln 2}{k} \quad \text{Eq. 6}$$

Kinetics of absorption/elimination follow either a first order (exponential) or zero order process.

First order kinetics: A constant fraction of the drug is absorbed or eliminated in a unit of time; the process is therefore directly proportional to drug concentration and applies to the majority of administered drugs.

Zero order kinetics: A constant amount of the drug is absorbed or eliminated in a unit of time, this process is independent of drug concentration. This process usually applies only when the drug concentration is high enough to saturate the absorption or elimination mechanisms. A constant rate i.v. infusion is an example for zero order kinetics as well as the elimination of ethanol [38, 39].

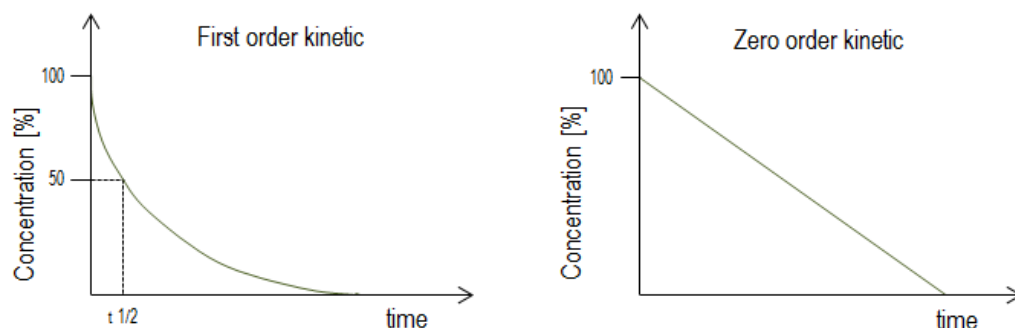


Fig. 1: Comparison of first- and zero order kinetics.

Plateau effect or steady state: If a drug is repeatedly administered in a certain time interval and elimination is a first order process, the plasma concentration [40] will approach a plateau value, also termed as steady state condition. The same applies for constant i.v. infusion (zero order process) with first order elimination [41]. While approaching the steady state there will be fluctuations in plasma concentration with a peak at each dose. If the half-life is shorter than the dosage intervals, high fluctuations of plasma concentration are the result. The shorter the time interval and the smaller the dose, the less fluctuations occur and the easier the maintenance of a constant level, which is crucial for some medications [38].

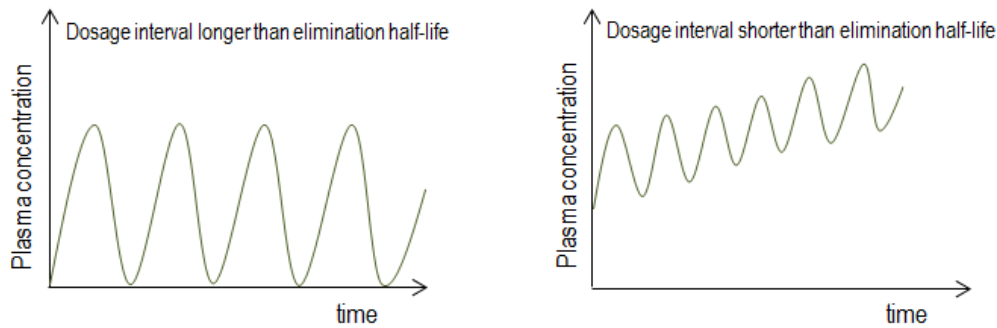


Fig. 2: Comparison of dosage intervals with high and small fluctuations

1.2.3. Distribution

After the drug has been absorbed (or just administered in case of i.v. application) it will be distributed throughout the body. Depending on administration route and drug properties such as lipophilicity, protein binding etc. the drug will either stay in the vascular compartment or be more or less widely distributed to the different tissues; the tendency of a drug for the former or latter can be evaluated by calculating its distribution volume [42].

Distribution volumes

In general terms, the volume of distribution is defined by the ratio of the drug amount in the body at a certain time and the plasma concentration at the same time.

$$V_d = \frac{\text{amount of drug present in body at given time } t}{C_{\text{plasma at given time } t}} \quad \text{Eq. 7}$$

As defined, V_d is just regarded as proportionality constant between the plasma concentration of a drug and its total amount in the body, i.e. to which extent the drug stays in the circulatory system vs. the amount which is distributed to the organs and tissues. As this proportionality constant has volume as dimension, V_d is named as volume of distribution [27]. It should be mentioned that due to the most diverse binding properties to tissue and proteins and the compartmental differences in distribution pattern of each drug, the volume of distribution cannot be regarded as a real volume but rather as an apparent volume, that provides the mathematical means of measure and an estimation of drug distribution in the body [42].

Drug distribution and volume of distribution are related but not the same, especially as several volumes of distribution can be calculated: Depending on measurement conditions, three distribution volumes can be differentiated: Directly after i.v. administration (volume of the central compartment V_C , see below), at equilibrium (steady-state) and at pseudo-equilibrium, from which the ones at equilibrium and pseudo-equilibrium are the most relevant ones for practical use. These different V_d are necessary, as the ratio between plasma drug concentration and drug amount in the body, yields different values for V_d in different states of drug distribution [27].

Also the compartmental drug distribution is important. If a drug stays in the plasma and does not partition into any tissues, a so-called one compartment model is enough to calculate V_d . This is rarely the case. The vast majority of drugs does partition into different tissues. Therefore, the intravascular space can be described as central compartment (including well perfused organs like heart, liver and kidneys) and the tissues as peripheral compartments. The extracellular space and intracellular space can be defined as compartments as well as certain tissues (e.g. fat) or organs [43, 44]. Also special compartments such as the CNS with restricted access by the blood brain barrier (BBB) [20] and the placenta are defined. Some drugs accumulate in certain compartments which can lead to toxicity [56]. The tendency of the drug of partitioning to some or all the tissues, the extent and velocity of doing so, binding to tissues or proteins and the “preference” of a certain compartment, all influences the volume of distribution of those drugs [40].

Directly after i.v. administration, plasma concentration of a drug is at its maximum (C_0) and before the start of all drug distribution and elimination, the

amount of drug in the body is defined as equal to the administered dose, so the initial distribution volume (V_C) is as following:

$$V_C = \frac{\text{dose}}{C_0} \quad \text{Eq. 8}$$

After the drug administration, the distribution and elimination start right away. If the drug distribution begins instantly afterwards, the body can be regarded as one homogenous compartment and V_C is the same as V_d . In most cases however this approach is not possible because of a delayed distribution where the plasma concentration of drug decreases faster than the total amount in the body; due to distribution of the drug throughout the body but not elimination. Referring to the definition of a distribution volume (s. eq. 7), V_d increases until the pseudo-equilibrium of distribution is reached, which is characterised by a balance between plasma (central compartment) and tissues (peripheral compartments). The only decrease of plasma concentration is now a result of irrevocable elimination, proportional to the total body clearance. In this situation the distribution volume reaches an asymptotic value, named V_{area} . So, during the distribution phase V_d can be seen as a time-dependent variable with a range starting from V_C and stretching until V_{area} .

$$V_{\text{area}} = \frac{\text{amount of drug in body during terminal phase}}{\text{plasma concentration during terminal phase}} \quad \text{Eq. 9}$$

As V_{area} can be only seen in relation to total body clearance, it is not the right distribution volume in the cases where clearance is zero/apparently zero, e.g. during i.v. infusion after achieving steady-state conditions. Here, the drug intake is equal to the loss through clearance, therefore as if no clearance occurred. In this situation the right distribution volume is the so-called V_{ss} , see equation below:

$$V_{\text{ss}} = \frac{\text{amount of drug in body at equilibrium}}{\text{steady-state plasma concentrations } (C_{\text{ss}})} \quad \text{Eq. 10}$$

All these distribution volumes are used to compute the drug amounts and loading dose. V_C and V_{ss} are often used for the prediction and prevention of critical doses. V_C itself can be used to calculate the initial maximum concentration of e.g. i.v. anesthesia or the estimation of the plasma volume of substances which do not leave the central compartment. V_{ss} can determine loading doses while V_{area} gives information about the residual amount of drug in the body during elimination phase. It should be noted, that for parenteral drug administration the

bioavailability factor F should be implemented in all the calculations. If F is unknown, the estimation of drug distribution becomes rather difficult and less exact [27].

1.2.4. Metabolisation

Metabolisation is a process happening to any drug applied to the body that reaches the circulatory system. First the drug is resorbed, which means that the concentration in the blood increases and the drug starts its effect. The last step is the elimination, where the drug is modified in a way that it can leave the body. Sometimes this process is already considered while designing a drug, so an inactive pre-drug can be applied that the body transforms to its active form [45]. Other times, the metabolisation multiplies the efficacy of a drug to such an extent, that it is toxic [46]. In every case, however, metabolisation is meant to serve the inactivation and excretion of the drug [45].

Metabolisation of drugs mainly occurs via the liver, gastrointestinal epithelia and kidney [45]. The liver is the first site, where all toxic substances (and all the drugs can be considered as such from the body's point of view) are collected and modified in a process which is called biotransformation. One feature of many drugs is that they are relatively unpolar (weak acid/weak base) and highly lipophilic, i.e. hydrophobic and poorly water soluble [21]. Therefore, in phase I of the biotransformation the substances will be polarized by reduction or oxidation. In phase II they will be conjugated with i.e. glucuronic acid and glutathion, or acetyl-, sulfate- or methyl groups are added. In this way, the substances are transformed from lipophilic to hydrophilic molecules, which is urgently necessary to enable the kidneys to excrete them along with the urine or the liver via bile [45].

First pass effect

For oral drug administration, the so-called first-pass effect is crucial in determining the bioavailability of a drug. The applied drug, absorbed in stomach or intestine, will be collected in the corresponding veins which meet in order to form the portal vein (*V. portae*). All the blood from this vessel flows directly to the liver before it is introduced to the systemic circulation [45].

This means that before reaching the target side, the drug passes the liver, where it

is partially metabolized, before it can develop any of the desired effects. Depending on the pharmacologic properties of the drug and individual enzymatic activity, this effect can reduce the drug's concentration by about 70 % or more [47]. Moreover, this effect is not just limited to the liver, but there is also a gastrointestinal first pass effect due to enzymatic metabolic activity of the gut walls [45, 48, 49]. The intensity of the mentioned effects not only strongly differ from individual to individual, but is also species-dependent, which can cause difficulty in predicting the behavior of drugs based on existing data [3, 4].

Enterohepatic recycling

Substances absorbed in the duodenum are transported through the portal vein firstly to the liver, as already mentioned. Part of the portal blood will be “used” for the secretion of bile back to the duodenum. Some substances are extracted from the portal blood by the liver, secreted into bile and again reabsorbed in the duodenum for up to 12 times or more [45]. These substances circulating between small intestine and liver are subject to enterohepatic recycling. This process is important for bile salts, bilirubin and different vitamins but also certain drugs can undergo enterohepatic recycling as e.g. antibiotics, NSAIDs and hormones [45, 50]. Factors determining whether and to which extent a drug will be recycled in such a way are among others drug characteristics, transport, biotransformation and intestinal absorption as well as genetic variations of the individual and health status [51]. Enterohepatic recirculation can prolong the pharmacologic effects of these drugs and drug metabolites [51] by increasing the toxicity if the substance remains active during circulation [46].

1.2.5. Elimination/Excretion

Excretion is the last step of the LADME processes, by which the drug is removed from the body. Effective excretion is only possible for hydrophilic molecules. Accordingly, either the drug itself is hydrophilic enough and can be excreted directly without undergoing metabolism (fraction unchanged) or (if it is more lipophilic) the drug is altered to a hydrophilic molecule during biotransformation. The water-soluble drugs and drug metabolites can now be excreted via the kidneys, but also via bile (and consecutively via feces), sweat, lungs and other routes. Renal drug excretion, however, is the main route for the majority of drugs. Renal excretion includes glomerular filtration, active tubular secretion and passive

tubular reabsorption [22].

Glomerular filtration: The kidneys filter a huge amount of blood per day which makes filtration an effective excretion route, but as the glomerular barrier blocks the passage of plasma proteins and generally large molecules, the drug fraction bound to any of these blood constituents cannot be filtered. Factors affecting the filtration of free drug in plasma are the renal blood flow as well as the glomerular filtration rate (GFR) [22].

Tubular secretion: In this renal excretion pathway substances in the plasma are actively transported into the tubular lumen by two transporter groups: OAT (organic anion transporter) and OCT (organic cation transporter). Drugs that are highly plasma protein bound can be excreted by tubular secretion [22].

Tubular reabsorption: As for other substances and body minerals (Na, Ka, Cl etc.), some (unionized) drugs are (passively) reabsorbed in the distal tubule and Henle loop and will be excreted in a following cycle [22, 52].

Summing up all three renal excretion processes, the total renal clearance (CL_R) can be calculated as following:

$$CL_R = f_u(GFR + CL_S) \times (1 - F_R) \quad \text{Eq. 11}$$

where f_u is the fraction unbound (free drug), GFR the glomerular filtration rate, CL_S the drug fraction secreted into the tubule and F_R the fraction reabsorbed in the Henle loop [40].

Further pharmacokinetic parameters corresponding to drug elimination and excretion are total drug clearance (Cl) and the biological or terminal half-life [52]. They can be defined as following:

$$Cl = \text{drug elimination rate constant (K)} \times V_d \quad \text{Eq. 12}$$

$$t_{1/2} = \left(\frac{0.693}{\text{clearance}} \right) \times V_{\text{area}} \quad \text{Eq. 13}$$

* valid for first order elimination drugs [28]

The influence of drug distribution on elimination is caused by the availability of the drug for the eliminating organs liver and kidneys which only have access to intravascular residing drugs. Therefore, the larger the distribution volume, the lower is the drug fraction inside the vascular system and consequently the elimination over time, resulting in a long half-life [27].

1.2.6. Limits of oral and parenteral drug delivery

In the previous few chapters some aspects of pharmacokinetics and pharmacodynamics referring mainly to general drug metabolism and metabolisation following oral administration were described. The goal was to provide not only overall understanding but also some important information why, although widely used, oral drug administration and its resulting systemic effects on the organism, are associated with several downsides such as e.g. low bioavailability of the drug resulting in inefficient treatment and potential toxic effects [53]. Some disadvantages of the oral route can be avoided with parenteral drug administration, but this method also has some critical drawbacks such as the invasive nature of parenteral (usually intravenous) drug administration. Also patient acceptance may be low due to pain associated with the injection procedure, and there is a risk of infection. To administer the drug parenterally, trained personal is required [53].

1.2.7. Advantages of transdermal drug delivery

Depending on the purpose of treatment, transdermal drug administration can offer a variety of advantages. It is painless, non-invasive, with a large surface easily accessible, increased bioavailability in the case of drugs undergoing extensive gastrointestinal metabolisation, and a rather stable pharmacologic profile with fewer peaks compared to other routes [1, 53]. To be able to consider transdermal drug delivery as an option and to get a deeper insight into the underlying mechanisms, a profound knowledge of the skin's anatomy, histology and physiology is needed.

2. Skin

2.1. Structure and physiological function

The skin is the largest organ in mammals. It is the most external layer of the body and its contact interface to the environment. The major task of skin is to separate the inside from the outside but also to enable controlled interactions with the outside world. It acts as a barrier against mechanical, chemical, biological, radiological and thermal influences, it keeps the homeostatic milieu of the body (regulation of water, mineral and vitamin household, maintaining body temperature etc.), it is surface for communication, receptor for pressure/strain/stress, pain, heat and cold, and it forms the first defense line of the immune system [54-57].

Its condition is directly linked to physical and mental health. The overall health status and many diseases can be recognized from the skin [54]. At the same time, skin problems also have a high impact on the general wellbeing of humans and animals [58, 59].

The skin can be differentiated in

- Subcutis (also known as hypodermis)
- Cutis (skin as such) with skin appendages and hair
- Skin modifications like nail, claw, hoof and horn

2.1.1. Subcutis

The subcutis or hypodermis [57] lies directly under the skin and is made from loose connective tissue, containing more or less large amounts of adipose tissue. It serves as isolator against cold, as energy storage, padding and also as a flexibly movable layer between skin and muscle. The continuous fat layers in the subcutis of pig and cattle are known as lard and tallow. Strands of tight connective tissue run through the subcutis to fix it to the underlying fascia and muscular tissue [54, 55]. The main cell type, the adipocytes are large (up to 100 μm) roundish cells with a lipid filled cytoplasm that pushes the nucleus against the cell membrane [60].

2.1.2. Cutis

The cutis defines the outer border of higher organisms which transitions to

mucosa at the natural body openings. It can be divided in

- Dermis and
- Epidermis

2.1.2.1. Dermis

The dermis is the base of the actual skin. It is the thickest layer of the skin. Variations in skin thickness result mainly from varying dermal thickness. Total human skin thickness including epidermis ranges from 0,05 mm at the eyelids to 1,5 mm at the palms and soles [55]. It is composed of a dense network of collagenous and elastic fibers, different cells and vessels. These are enclosed by the gel-like so-called ground substance, consisting of water binding mucopolysaccharids, also named proteoglycans. They belong to a group of glycosaminoglycans with their major representative being hyaluronic acid. In humans, two layers of the dermis can be structurally differentiated [54, 55].

- Stratum reticulare
- Stratum papillare

The thicker **stratum reticulare**, adjacent to the subcutis, is made of dense, tight connective tissue and is rich in collagen and elastic fibers. They provide flexibility and firmness to the skin, making it resilient to mechanic stress. A network of lymphatic capillaries is located in the deeper part of the stratum reticulare as well as epidermal appendages like hair roots, sweat glands and sebaceous glands [54, 55, 61]. Pacini corpuscles, registering pressure, can be found in the deeper parts of dermis as well as in the subcutaneous tissue [55].

The thinner **stratum papillare**, directly connected to the epidermis, comprises a high amount of cells, capillaries and lymph vessels. The main cell types of both stratum reticulare and papillare are fibroblasts. They produce and secrete procollagen and elastin which are transformed to the aforementioned fibrous networks. Collagen fibers provide especially tensile strength and resistance to shear stress while elastic fibers help resisting deformation and returning the skin to its normal shape [54, 61]. The other important cells are mast cells. They play a crucial role in the mechanism of chemotaxis in allergic and inflammatory reactions. Their granules release cytokines and other pro-inflammatory mediators upon activation by surface antigen binding [62]. They also influence cell

proliferation, the production and remodeling of collagen and the formation of scar tissue after injury [63].

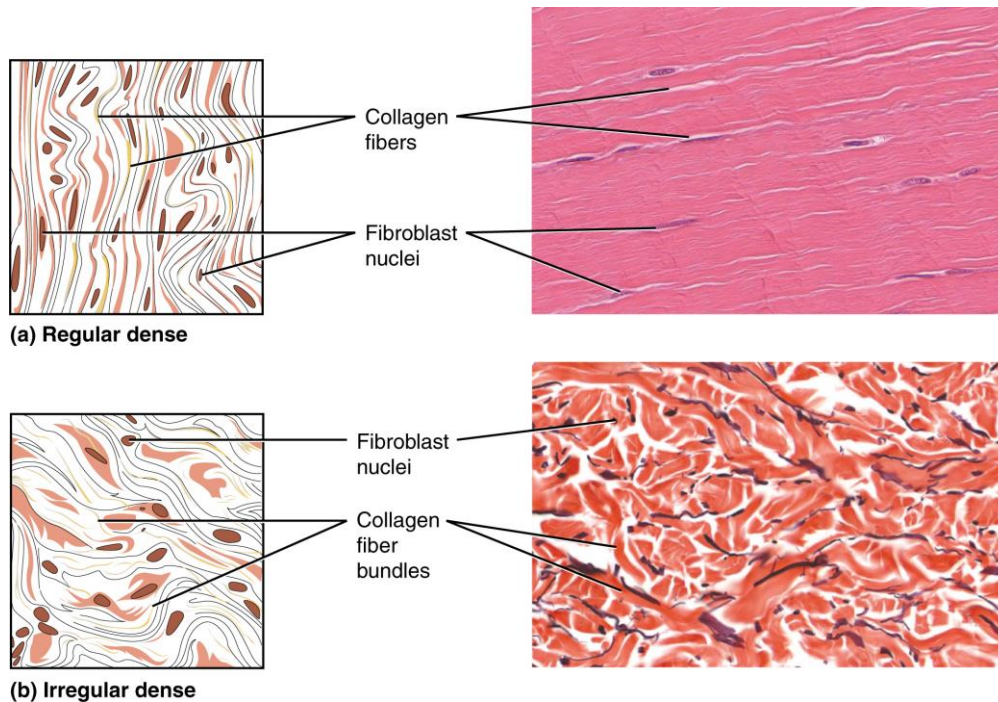


Fig. 3: Connective tissue: a) regular and b) irregular dense connective tissue

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The border between the stratum papillare and the epidermis is referred to as dermoepidermal junction and characterized by an undulating basement membrane [55]. In humans, the contact surface to the epidermis is hereby highly expanded by the formation of numerous fingerlike projections of the dermis, the papillae, which are interdigitated with surrounding papillae from the epidermal side, also referred to as “rete ridges”, see fig 4 [54, 57]. This strengthens the connection between both layers, by also increasing the surface area for exchange of oxygen, nutrients and waste materials by facilitated diffusion from the vascularized dermis toward the avascular epidermis and the reverse. Sebaceous glands can be found here as well as in the stratum reticulare [54]. Meissner corpuscles are also numerous located here, responsible for the detection of light touch [55].

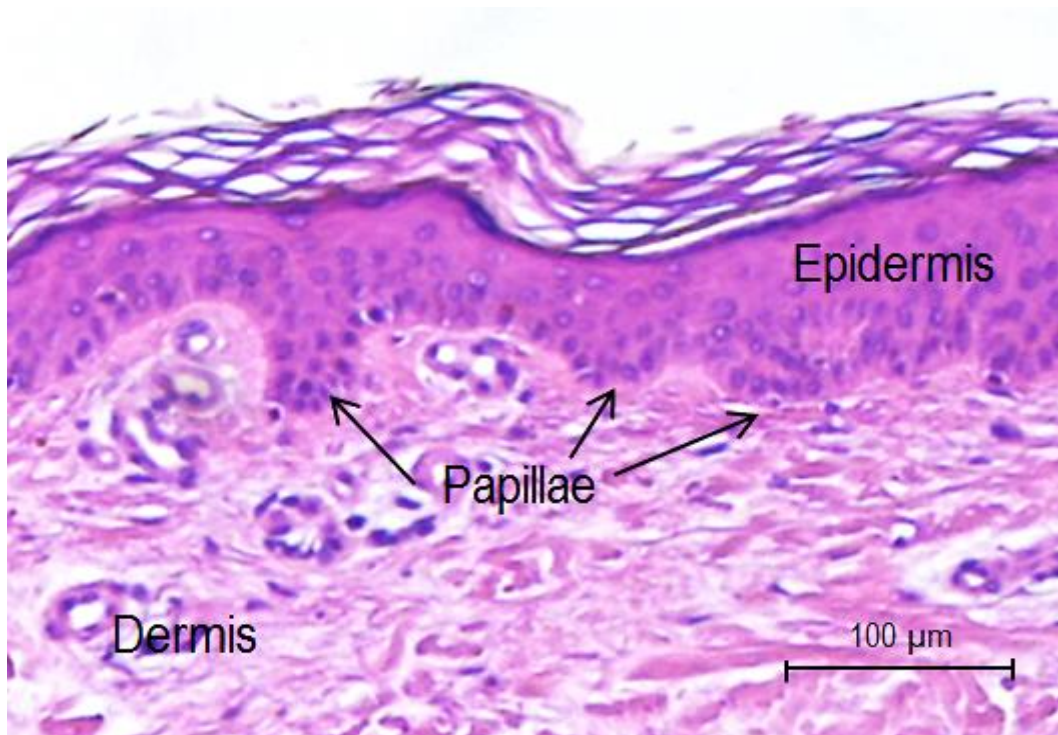


Fig. 4: Histologic section of dermoepidermal junction and papillae, stained with H.E.

With friendly permission of Kai Emrich, Institut für Pathologie Saarbrücken-Rastpfuhl

2.1.2.2. Epidermis:

- The epidermis is a multi-layered keratinising squamous epithelium. It is made from several different layers in which the skin cells, the keratinocytes are built, multiplied and differentiated. From the deepest layer they get pushed toward the outer layers by new cells coming after them and are shed after ca. 29 days [54, 55, 60, 61]. The average thickness of the human epidermis is around 100 µm [60] with similar values for porcine epidermis [64]. Canine epidermis, however, ranges from approximately 25 to 45 µm (depending on breed and body region), thus being significantly thinner [65]. As the epidermis is avascular, nutrients and oxygen reach only the bottom layers which leads to cell death in the upper epidermal regions [54, 55, 61]. Following layers can be generally differentiated although differences between species and body region are observed [64]:
 - Stratum basale
 - Stratum spinosum
 - Stratum granulosum
 - Stratum lucidum

- Stratum corneum

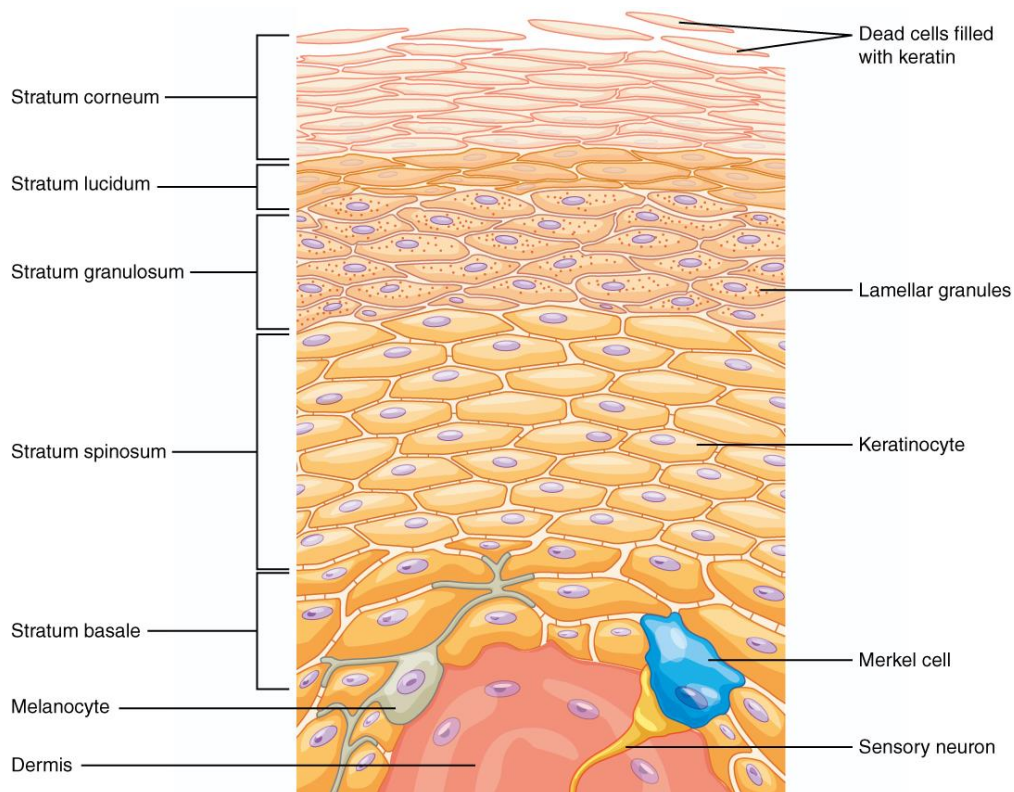


Fig. 5: Schematic diagram of the different layers of the epidermis

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Stratum Basale (Basal cell layer)

The stratum basale, together with the stratum spinosum and the stratum granulosum also known as Stratum germinativum (meaning living, proliferating layer, equal to the term “viable epidermis”), is the deepest layer of the epidermis. It is directly superficial to the stratum papillare of the dermis and part of the dermoepidermal junction. From this single to triple layer of cubic cells (6 to 10 μm diameter), anchored to the basement membrane by hemidesmosomes, new skin cells are built. These cells, called keratinocytes, migrate by differentiating gradually towards the stratum corneum. The basal cell layer is the location of melanocytes, which produce the pigment melanin that gives the skin its color and helps protecting the skin against UV radiation [54, 55, 61].

Stratum spinosum (Prickle layer)

This layer is characterized by several (5 to 15) layers of polyhedral, larger

keratinocytes (10 to 15 μm diameter), connected by glycoprotein structures called desmosomes [57, 60]. The cells develop a flattened appearance while they are filled with lamellar granules and keratohyalin masses. The mitochondria and nuclei start showing signs of degradation [57].

Stratum granulosum (granular layer)

In the stratum granulosum, shortened as SG, the flattened keratinocytes (ca. 25 μm diameter) are further differentiating into corneocytes. The key transformation to form the final SC takes place here. The name of the SG is derived from the granules that appear in this just a few cell layers (1 to 3) thick sheet [56, 60]. Two kinds of granules are produced by the cells; keratohyalin granules containing keratin precursors and lamellar bodies (also known as Odland bodies) containing lipids. In the course of the transformation, the cytoplasm vanishes, the nucleus is digested and the keratin filaments start forming microfibrils. The cell membrane is superseded by a so-called “cell envelope” made from cross-linked protein. The lipids of the lamellar bodies are thus released into the intercellular space [56]. Keratin belongs to the protein family of intermediate filaments which are part of the cytoskeleton of nucleic cells. This tough fibrous protein is a crucial structural compound of skin, nail and hair. Keratin type I is acidic while a second type is neutral to basic. This enables the two helical proteins to interact with each other to form a so-called “coiled coil” structure and aggregate to microfibrils which lie parallel to the skin surface and strengthening the corneocytes [56]. The desmosomes of the stratum corneum are modified by addition of another protein and also called corneosomes [56, 57].

Stratum lucidum (clear layer)

The Stratum lucidum has its name from the light breaking properties of young differentiating keratinocytes. It only occurs at the fingertips, palms and soles of human or at the footpads and nose of members of dog and cat family and comprises three to five layers of squamous keratinocytes, devoid of nuclei [54].

Stratum corneum (horny layer)

The Stratum corneum, shortened as SC, is the top, outermost layer of the epidermis and the primary barrier against any outside influence. It comprises, depending on species and body site, ca. 9-50, in average (human) 12-16 layers of

non-viable, squamous, hexa-or pentagonal keratinocytes or corneocytes with a diameter of 29 to 40 μm [56, 60]. They are constantly shed and replaced by keratinocytes coming up from the deeper sections. MCM (membrane coating material), as intercellular matrix, and desmosomes keep the corneocytes together [54, 56]. In this way, the SC is often referred to as a conceptual model of a wall of bricks with mortar in between them, the “bricks and mortar model” [56]: According to this model the keratinocytes are symbolized as bricks and the MCM as mortar. The latter contains epidermal lipids such as ceramides, cholesterol, cholesterol esters and fatty acids. This lipid intercellular matrix forms a semipermeable water barrier, keeping needed skin moisture inside, an absolute necessity for land living creatures [54-56]. Lipophilic substances on the other hand penetrate the epidermis easily. Its function is essential for an intact skin barrier. The healthy SC can protect the organism against invading microorganisms, irritating chemicals and allergens [56]. A morphological subdivision of the SC into the deep stratum compactum with densely packed keratinocytes and the superficial stratum disjunctum where the cells are being shed is possible [60].

Epidermal cells

The cells in the epidermis are 85% keratinocytes. The other 15% are melanocytes, Langerhans cells and Merkel cells, situated in the viable epidermis. While melanocytes protect against UV radiation as mentioned above, Langerhans cells, originating from the bone marrow, belong to the MPS (mononuclear phagocytosis system) of the cellular immune system and get activated by viral infections, skin tumors and contact allergies [55]. By ingesting the antigens and breaking them down to small peptide fragments, the Langerhans cells bind the remains of the antigen to major histocompatibility complexes (MHC) to be recognized by lymphocytes (T-cells) in order to activate the immune response [55, 60]. Merkel cells are tactile receptors that transfer information about touch to the intra-epithelial nerve ends [54, 55]. Free epithelial nerve ends in the basal layer transmit pain while Krause bulbs recognize cold and Ruffini corpuscles detect heat, both are located in the superficial epidermis [6].

2.1.2.3. Epidermal Appendages

Epidermal appendages are epithelial structures located inside the dermis, lined

with epithelial cells which are able to divide and differentiate. These cells are a crucial reserve of epithelial cells for re-epithelialization after damage or destruction of the overlying epidermis [54, 55]:

- Sebaceous glands
- Sweat glands
- Hair follicles

Sebaceous glands

Sebaceous or holocrine glands are spread over almost the whole body. They are always connected to one or several hairs [54]. Being most concentrated and largest in size in the face and scalp of humans and the chin of cats, they are the site of origin of acne. The secretion of sebaceous glands, the sebum, is a mixture of complex oils including triglycerides, wax esters, cholesterol and its esters, squalene and more [55]. It gets mixed with the secretion of apocrine glands and serves the purpose of forming a protective lipid layer on the skin to keep hair and the stratum corneum smooth, healthy and more impervious to moisture [54].

Sweat glands

Two types of sweat glands can be differentiated, which secrete either apocrine or eccrine. Apocrine sweat glands are associated with sebaceous glands and release a slightly alkaline liquid which is responsible for the individual smell of any human or animal [54]. The mammary gland is considered as a modified and highly specialized apocrine gland [55]. Eccrine sweat glands are especially frequent in higher primates and secrete a relatively acidic watery liquor, the sweat [54]. By evaporation, sweat has a cooling function on the body and prevents overheating due to environment temperature, sun exposure and effort. Its release is triggered by exceeding the set body core temperature [55]. Thermoregulation in most mammals other than horses and primates does normally not involve sweating, therefore they do not have eccrine sweat glands [66]. Sweating in species such as human and horse can be also induced by catecholamine release (e.g. adrenalin, noradrenalin), as a result of elevated stress levels [67-69].

Hair follicles

The intradermal hair root or hair bulb is a part of the hair follicle which is built by an epithelial and a fibrous layer derived from both epidermis and dermis, which

are also known as hair root sheath [54]. They are ubiquitously spread over the body. Sebaceous and also sweat glands often open into the hair follicles instead of directly toward the skin's surface. The entity of glands and hair is named a pilosebaceous unit [55]. While humans, horses and cattle have hair follicles surrounding one single hair [66], dogs and cats have compound hair follicles, meaning that their hair grows in bundles. For the dog it is usually one primary and up to 14 secondary hairs (see next section "hair") in one follicle whereas cats have single primary follicles with up to five surrounding compound follicles with around three primary and up to 12 secondary hairs in each of them. The density of hair follicles can vary from around 40 to 60 follicles per cm^2 (human and pig) over 900 follicles/ cm^2 (cattle) to amounts of 10,000 follicles/ cm^2 in merino sheep, again with considerable variation between body regions [64].

Hair

Hairs are long, thin and elastic filaments made from epidermal keratin. They are exclusively characteristic for mammals and cover their bodies in various length and thickness. They protect against cold, humidity and can prevent or minimize injury. While in normal condition the hair follicles are oriented inclined to the skin surface, they can be raised vertically by a strand of smooth muscle, the *M. arrector pili*, which connects the follicle to the superficial dermis (causing e.g. goosebumps) [55]. Hair color is determined by granules of melanin within the hair [54]. There are three main kinds of hair: Vellus hair, intermediate hair and terminal hair. The gentle, almost transparent vellus or "wool" hair is spread all over the human body, excluding areas like palms, soles and lips (so-called glabrous skin) [70] and has for humans an average diameter of 16-18 μm [56]. Terminal hair on the other hand is stronger pigmented and thicker; between 57-68 μm in diameter [56, 70]. Scalp hair, eye brows, eye lashes, axillary and pubic hair are examples of terminal hair. Hair of arms and legs lies in between in thickness and color and is therefore categorized as intermediate hair, with a thickness of 28-42 μm diameter [56, 70]. This very same differentiation applies to all mammals but with the difference that many of them have a higher density of vellus hair in total, covered by another layer of terminal or primary hair [54].

The hair root is only completely developed during the anagen or growing phase (see below). The follicular epithelium is surrounded by the root sheath and at its bottom is thickened to form the hair bulb enclosing the hair papilla [54]. The

continuation of the hair bulb is the hair shaft. The hair shaft separates from the hair follicle and its outside part protrudes from the skin surface. The keratinized hair can be differentiated into three segments; namely the hair cuticle covering the inner layers with flat cells, overlapping each other similar to the scales of a fish, then the hair cortex, containing bundles of keratin and structural lipids and on the inside of the hair fiber the medulla, an open area at the center [54, 71, 72]. Again this structure is similar for all mammals but shows enough morphologic differences to distinguish human hair from animal hair by analyzing the scale pattern of the cuticle and the ratio of medullary width to diameter of the hair [72].

Hair growth is a cyclical process with three repeating stadiums. The anagen phase is characterized by growing hair. The catagen phase is the transition time from anagen phase to telogen phase. In the following telogen phase, the hair follicle is resting. The duration of the anagen phase is directly proportional to the length of the created hair. At every place of hair covered human body, all three phases are visible next to each other. They can differ in duration depending on anatomic location, genetics and individual health status [55] Animals (mammals) often exhibit a seasonal hairgrowth, induced by factors such as the length of daylight or temperature [73].

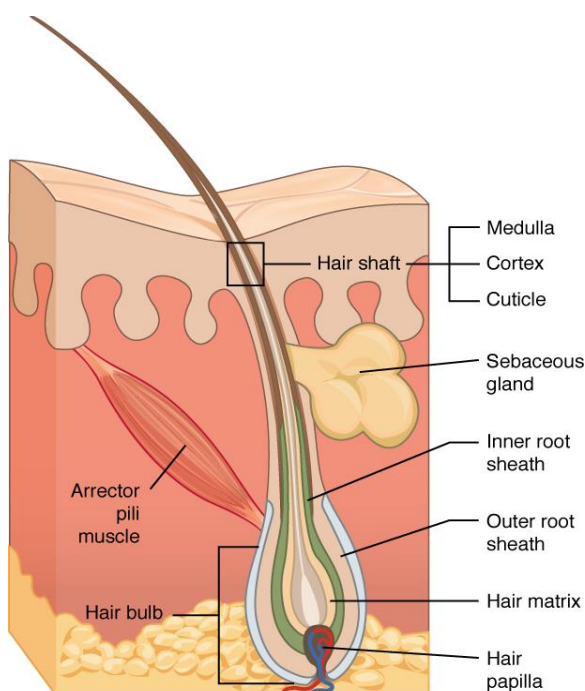


Fig. 6: Schematic drawing of the different sections of a hair *in situ*

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2.1.2.4. Skin modifications

Besides the already mentioned skin modification of skin-associated glands and hairs (epidermal appendages), also other structures are considered part of the skin.

- Footpads (mostly animal)
- Nails/claws/hooves (human and animal)
- Horns (obviously animal)

Each of them is an adaptation of epidermal, dermal and hypodermal structures to meet the needs of different species. While footpads are characterized by an especially strong upholstery of subcutaneous fat, nail/claw/hoof and horn are formed by highly specialized keratinocytes [54].

Embryologically, subcutis and dermis are of mesodermal origin while the epidermis and its appendages/modifications are derived of ectodermal origin [60].

2.1.2.5. Skin barrier and acid mantle

As explained before, the skin barrier is mainly formed by the SC, consisting of keratin and lipids, describable with the aforementioned bricks and mortar model. The lipids are hereby not homogenously dispersed in the extracellular space of the SC but organized into multiple sequestered lamellar membrane bilayers. The degradation of corneodesmosomes leaves spaces in these lamellar lipids. This way, in a healthy SC under normal conditions, a discontinuous system of tunnel-like structures, the so-called lacunae, is formed. Subjected to certain treatments (e.g. occlusion, iontophoresis, sonophoresis, and prolonged hydration) these lacunae can temporarily extend and enlarge and create a continuous and permeable system, like pores, an effect that is exploited in transdermal drug delivery (see chapter 3). A further feature of the SC is its function as a very precise biosensor; already a small change of air humidity can up- or down-regulate the synthesis of epidermal lipids, filaggrin (main component of the cornified envelope of SC cells) and DNA, and also promote inflammation. This is possible via connections down to the viable cells of the epidermis [74].

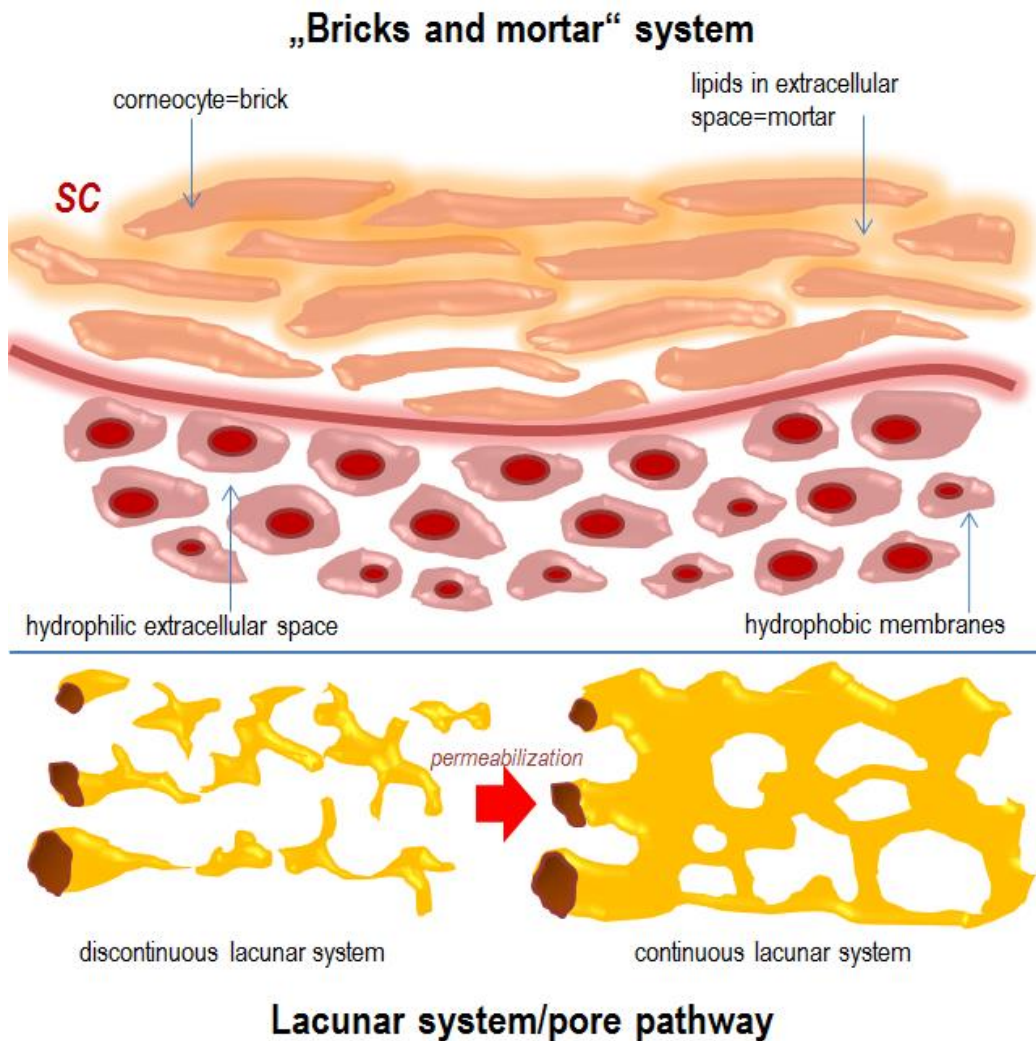


Fig. 7: Visualization of bricks and mortar model and lacunar system model

Another aspect that accounts to the SC barrier is the so-called acid mantle [56]. The mortar lipids are made up of three main types: Ceramides, cholesterol and free fatty acids, in the ratio 1:1:1. Their very composition and distribution provides the high hydrophobicity and the acidic pH of the SC, which usually varies between 4 and 5.5 (human). This pH acts as an additional protection against bacterial colonization of the skin. Disruption in the ratio of these lipids leads to an impaired barrier function due to alteration in the membranes [56, 74].

2.2. Unphysiological condition

The skin, especially the stratum corneum, can only fulfill its natural protective tasks as long as the homeostasis of the skin and skin associated milieu remains undisturbed. If due to any internal or external influences the sensitive equilibrium is impaired, the skin barrier becomes leaky and leaves the organism vulnerable to the hostile outside world.

2.2.1. Damaged skin barrier

Whatever the underlying causes for an affected skin barrier are, they can frequently be broken down to three complex conditions which are to a high extent mutually dependent. These are:

- Dryness
- Irritation due to chemicals/radiation
- Inflammation

2.2.1.1. Chemical irritation and dryness

Chemical irritation of the skin is usually associated with dryness, as the damaged skin barrier cannot keep moisture inside anymore. One common example for skin irritants are surfactants as ubiquitous components of most washing and cleansing products, i.e. soaps, lotions, shampoos etc. They are surface-active molecules comprised of a hydrophilic head group and a hydrophobic tail portion (amphiphilic molecule). Their mode of action however can cause serious damage to both the bricks and mortar of the SC barrier. Surfactants bind to proteins by changing their structure and have been proved to also bind to the SC and cause swelling. Surfactant effects on the lipid mortar include removal of lipids as well as alteration of their composition by disordering their lamellar structure. Further effects focus on the protective acid mantle of the skin: The natural SC pH of 4.0 to 5.5 is raised by washing with soap to 7.5. While washing once in a while does not cause any further harm and the skin is repaired in short time, repeated washing can exceed the buffering and repairing ability of the skin, i.e. between the washing cycles the pH does not return to its normal value. The damaged SC barrier causes loss of water. Therefore, the skin gets dry and dry skin is not flexible and pliable anymore but stiff and brittle. This leads to cracking (fissuring) of the skin which is now open and permeable for other chemicals, harmful bacteria etc. which further challenge the disrupted skin. Low hydration status of the SC disturbs also the normal desquamation, resulting in shedding large flakes instead of normal cells, thus increasing the surface area for bacterial colonization. Surfactants can also trigger the liberation of pro-inflammatory cytokines by interacting with the keratinocytes in the viable layers of the epidermis. This can cause an overstimulation that leads to hyperproliferation of the skin which does not leave enough time for the complex processes necessary for the formation of a fully functional SC [56]. Organic solvents such as ethanol damage the SC barrier in a

similar way to the surfactants; by delipidization. Acids and alkali (bases) cause a conformational alteration of skin protein and damage the epithelium of the sweat gland ducts, thus increasing the porosity of the skin. The result can be an immense water loss of up to 350 % following NaOH and Na₂PO₄ application, both pH 12, [75] as well as vesicles and inflammation. UV irradiation is another irritant to the skin, inducing epidermal hyperplasia, alteration in lipids, erythema and inflammation [75].

2.2.1.2. Inflammation

Skin inflammation can be induced by many factors. UV irradiation is one of the main sources of skin inflammation in humans, along with skin injury, microbial invasion and allergies. In contrast, UV damage is only seen in sparsely haired and nonpigmented skin such as the eyelids, ear tips or the nose in animals. In many inflammatory skin disorders, the process may start with pruritus. Upon scratching, the skin is subjected to mechanical trauma and barrier disruption and reacts with inflammation which, in turns, enhances the itching, thus creating a vicious circle which is the harder to break the longer the situation exists (see figure 9).

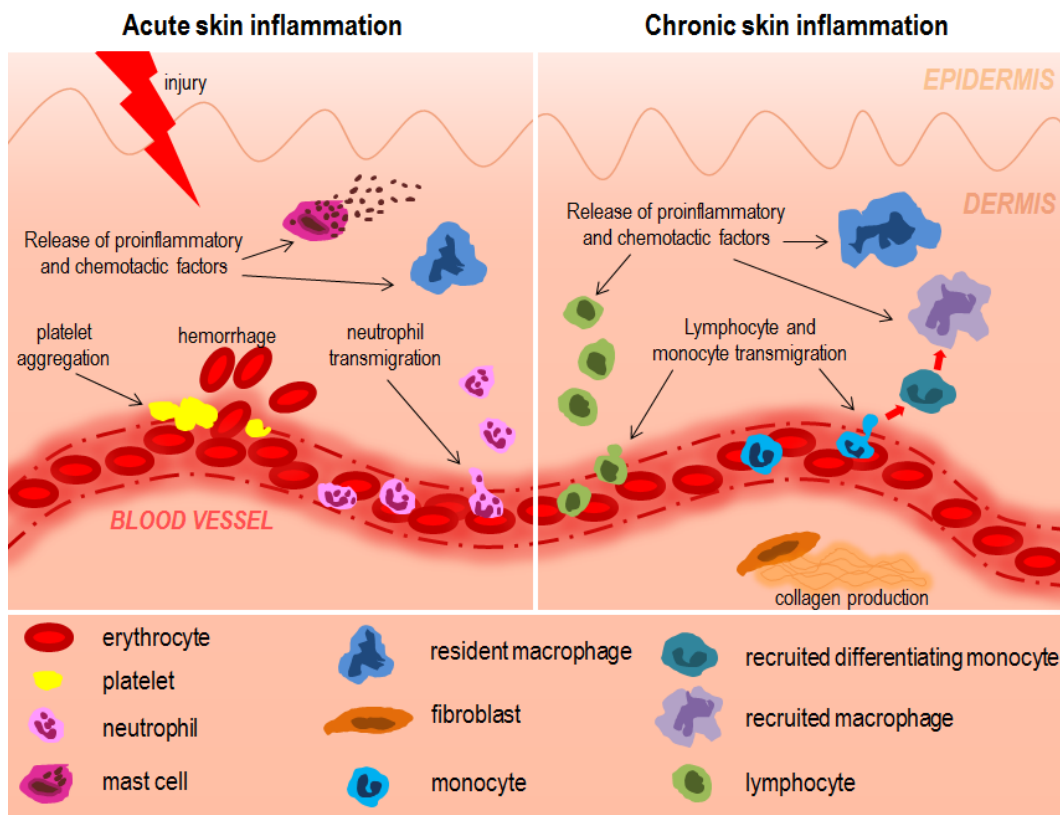


Fig. 8: Acute vs chronic skin inflammation.

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One example of such an amplification cascade is atopy-like/atopic dermatitis. Genetic factors and/or environmental influences lead to an increased inflammation with production of allergen-specific IgE, a preponderance of T helper 2 cells with release of cytokines such as IL-4, IL-5, IL13 and IL-31. The subsequent pruritus leads to scratching. By scratching the itching skin, primary proinflammatory cytokines such as IL-1 α , IL-1 β , TNF- α and chemokines are produced which together with adhesion molecules direct the chemotactic recruitment of leukocytes to the skin, including dendritic cells. These prompt mast cells to release histamine via antigen-specific IgE complexes. The disturbed skin barrier opens way for bacterial colonization of the inflamed skin, releasing more inflammatory mediators, e.g. staphylococcal enterotoxins, which summons further immune cells to the site (memory T-cells, possible involvement of eosinophils) which sustain the inflammatory cycle [77]. This activation of both the innate and the adaptive immune system triggers mass production of reactive oxygen species (ROS) as a result of phagocytosis. These free radicals usually serve defensive purposes but if they are produced in excess, so that the cellular scavenging system cannot cope with their amount, a radical chain reaction may be started, leading to considerable damage of host cell membranes. Due to the high amount of polyunsaturated fatty acids (PUFAs) and continuous exposure to UV light, which both contribute to the forming of ROS, the skin is especially susceptible to damage by free radicals, further promoting inflammation and inflammatory diseases [78].

Inflammation is macroscopically defined by five major or “cardinal” signs, namely: calor (heat), rubor (redness), dolor (pain), tumor (swelling) and functio laesa (loss of function), all of which are also present in skin inflammation.

Skin diseases

For the interested reader are some examples of infectious/inflammatory cutaneous diseases such as Acne vulgaris or Canine atopic dermatitis described in the Appendix.

3. TDD (Transdermal drug delivery)

Transdermal drug delivery is, as such, not a new concept as it has been carried out for thousands of years in the form of creams and ointments placed on skin [1]. However, the purpose typically was to treat the skin or underlying tissue. It was less commonly used to administer drugs for treatment of systemic diseases. Compared to other ways of drug administration, delivery via skin has numerous advantages. As largest organ of the body, the skin offers a vast surface that can be employed for percutaneous absorption. Drug administration is non-invasive, painless and does not require medical personnel in contrast to e.g. injections. It can be applied repeatedly to the same region without considerable irritation and is thus suitable for long-term treatment [2]. Transdermal delivery avoids side effects and also drug metabolism in the gastrointestinal tract (s. first-pass effect) [83, 88]. In many instances, lower dosages are needed than with oral intake, contributing to the lower incidence of adverse effects. By circumventing gastrointestinal metabolism, a more sustained and controlled drug release with stable plasma levels is possible [2], a feature which is generally not provided by both oral and parenteral administration, but may be vital for some patients. In many cases, the corresponding products and procedures are inexpensive in contrast to their counterparts for other administration routes [1]. However, the physiologic function of the skin, namely of the SC, to keep exogenous substances outside, is so well designed that the epidermis was considered an impregnable barrier to TDD for a long time.

It took until 1979, when the first transdermal patch was approved by the FDA, delivering scopolamine for the treatment of motion sickness. Approximately ten years later, with the introduction of nicotine patches for people wanting to quit smoking, TDD gained more attention and popularity and was finally recognized as a profitable research area. Other patches for various diseases entered the market during the following years. They can be described as first-generation transdermal delivery systems which are based on diffusion [82,83]. Testosterone patches against hypogonadism (1993), fentanyl against chronic pain (1990) or estradiol/norethidrone against menopausal symptoms (1998) can be listed as examples [1]. However, the substances employed for these first-generation systems were strongly limited due to constraints based on their physicochemical properties. To overcome the skin barrier without further enhancement, a

molecular weight below 400-500 Da, small particle size, high lipophilicity with a corresponding logP value and therapeutic efficacy at low doses (5-10 mg/d) [89] are required [1, 2].

The second generation of transdermal delivery systems can be characterized by employing permeation enhancers to expand the scope of drug candidates. Increasing delivery into and through the skin had to be balanced against injury to the tissue [1]. Permeability enhancement can be achieved by chemical enhancers as the most conventional method but also by other techniques like iontophoresis and ultrasound.

In the third generation, combinations of chemical enhancers as well as biochemical enhancers were tested (e.g. peptides). With electroporation and microneedles, the SC barrier could be evaded while with thermal ablation or microdermabrasion it was completely removed [1]

A fourth generation combining wearable devices and drug delivering patches connected over a feedback loop, opening the way for personalized therapy, is currently under research [2].

3.1. Percutaneous absorption

The term percutaneous absorption refers to the permeation of a substance through the SC and epidermis into the viable layers of the dermis and from there into the circulation (local clearance), hence describes an *in vivo* process. Permeation is the first part of this process, the diffusion across the skin [79]. Penetration can be viewed as a part of permeation where a substance does not pass through a layer but enters it and gets stuck inside. These two latter terms can describe *in vitro* processes and will be used in this context. As, *in vivo*, permeation is the rate controlling factor and not clearance, determination of permeability *in vitro* is thought to reflect *in vivo* conditions well enough to predict the bioavailability of drugs via this method [79].

3.1.1. Skin permeation

When testing a drug candidate or formulation intended for percutaneous application, usually an *in vitro* permeation study will be conducted during the process [80]. The substance is screened for its permeation properties and how it

can be modified.

3.1.1.1. Selection of (skin) membrane

For *in vitro* permeation studies, different membranes can be used. Employment of human skin is preferred for human application but also substitutes from animal skin, artificial skin tissue or reconstructions can be taken. Choice of skin and skin preparation strongly depend on intended application and tissue availability [15] . Generally, following possibilities exist, which will be described in detail in chapter 4 *In vitro* skin models:

- freshly excised skin from surgery or cadaver skin
- stored (frozen/thawed) skin
- full-thickness (FT) skin
- split skin which will be dermatomed to the desired thickness
- skin with removed SC layers by tape stripping
- heat separated epidermis (HSE)
- trypsin isolated SC
- artificial skin
- reconstructed epidermis
- animal skin from pig, rat, mouse, guinea pig, snake and other species

3.1.1.2. Diffusion cells

For measuring the skin permeability, the selected skin membrane will then be placed in a diffusion cell. Although a variety of diffusion cells exists for this purpose, the principle is always the same. They are made of stable and inert materials such as glass or steel [79] and consist of two chambers, the donor and the receptor compartment. The membrane is placed between the two compartments with the SC facing towards the donor compartment where the API is applied. The other compartment is filled with receptor solution. Over a defined time, the API will permeate through the membrane and can be quantified in the receptor solution.

From all different models of diffusion cells, two designs are most common. One is the side-by-side diffusion cell, a design employed by Wurster *et al.* [81], Flynn and Smith [82], Southwell and Barry [83] and many others [80]. More common is

the diffusion with vertical compartments with the donor compartment on the top cell mimicking *in vivo* conditions. This design is more suitable to variation of the donor vehicle, enables finite and infinite dose experiments and offers better control of atmospheric conditions [80]. The Franz diffusion cell is a very popular example of a vertical diffusion cell (s. figure 9). Different types of Franz diffusion cells offer a diffusion area of 0.1 to 39 cm². The volume of the receptor chamber is generally defined whereas the volume of the donor can be varied [84]. The membrane is fixed between both compartments using clamps. The temperature of the tissue and the receptor fluid should usually be maintained at $32 \pm 1^\circ\text{C}$ which can be achieved with a water bath system [79].

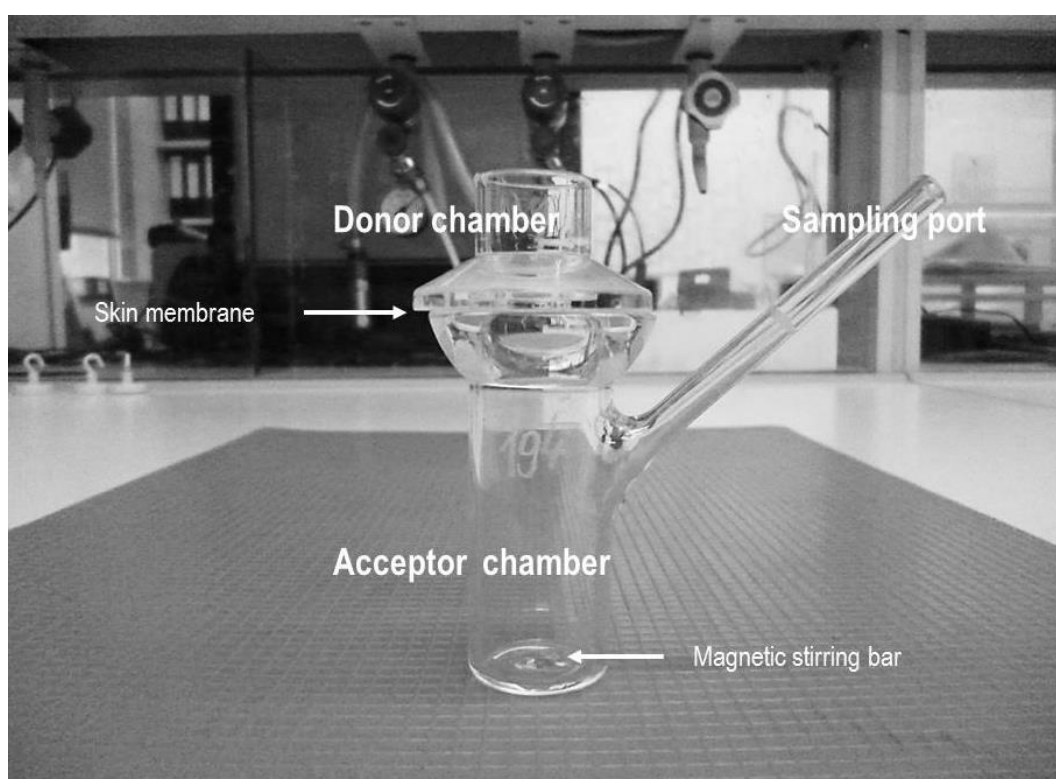


Fig. 9: Depiction of a regular Franz diffusion cell

3.1.1.3. Receptor fluid

As receptor fluid generally an isotonic buffer solution with a pH of 7.4 is chosen, e.g. PBS or KRB. Its solubility and stability regarding the corresponding experimental conditions has to be ensured. It must be assured that it does not compromise the integrity and permeability of the skin [84]. The thermodynamic activity of the drug in the receptor solution must not be higher than 10 % of its activity in the donor liquid that the permeation proceeds in the desired direction [79]. It is inevitable to constantly stir the receptor fluid in order to distribute the

API homogeneously inside and avoid local concentration peaks. Agitation of the receptor fluid reduces static diffusion boundary layers and thus increases permeation rates. The aim is to maintain so-called infinite sink conditions throughout the complete experiment [80]. While for hydrophilic drugs aqueous receptor fluids such as the aforementioned buffers are preferred, the addition of solubilizers, i.e. BSA or isopropyl myristate, might be required for lipophilic compounds [79, 80].

3.1.1.4. Donor solution

Occlusion vs. Open application: Depending on the product to be tested, the permeation experiment can be conducted under occlusion or as open application. Occlusion is achieved by the coverage of the donor compartment with a moisture-impermeable membrane which can prevent evaporation of the drug formulation/solution or hydrate the SC for the experiment [79].

Finite vs. infinite dose: The API can be applied as finite or infinite dose. Infinite dose is defined as an amount of more than 10 μL drug solution per cm^2 skin while for a finite dose the amount of drug solution per cm^2 skin is less than 10 μL . By infinite dosing, the velocity of permeation can be measured as so-called permeation coefficient. By finite dosing, the focus lies on the evaluation of the distribution of the drug within the skin and also the receptor fluid.

3.1.1.5. Experimental procedure

It is important to assess the skin membrane for its integrity before the study. Next to macroscopic examination this can be done by conducting a separate permeability experiment with a validated quality marker, e.g. caffeine. Usually, the number of required specimens is excised from the used membrane directly before the experiment, generally circular pieces of variable diameter according to the employed diffusion cell. They are fixed between donor and receptor compartment with a clamp, receptor fluid and API are added subsequently. The whole set up can then be placed in a water bath and magnetically stirred. A typical permeation experiment has a preset duration of 24 or 48h with defined sampling time points where a small amount of receptor fluid is sampled via the arm of the diffusion cell and then analyzed qualitatively and quantitatively by e.g. HPLC (High Pressure Liquid Chromatography), scintillation counting or any other suitable method [84].

3.1.1.6. Permeability analysis

Generally, the transport of molecules across any barrier can be quantified by flux which quantifies the amount of moving particles as a function of time as described in Fick's first law:

$$J = \frac{m}{At} \quad \text{Eq. 14}$$

with J being the flux (usually as mg or mol/cm²/s) of a mass or amount m of a compound over an area A during a time t. If within the compartments of the diffusion cell there is no other concentration gradient than that through the barrier, i.e. skin membrane, then Fick's law can be modified as

$$J = P (C_{\text{donor}} - C_{\text{receptor}}) \quad \text{Eq.15}$$

where P is the permeation coefficient, C the concentration in the donor and receptor compartment and J the flux from donor to receptor compartment. P is often described as P_{app} as it is an apparent coefficient due to simplifications and depending on the experimental conditions. Assuming that the transport across the membrane and the concentration in the donor chamber is constant and the concentration in the receptor chamber is so much smaller than in the donor that it can be considered practically zero, then the equation can be further simplified as follows:

$$J = P_{\text{app}} C_{\text{donor}} \quad \text{Eq. 16}$$

and transformed to:

$$P_{\text{app}} = \frac{J}{C_{\text{donor}}} \quad \text{Eq. 17}$$

With this equation, the permeability of a drug substance through a certain barrier can be calculated. Several substances can be compared with each other and categorized in permeability classes [85] (BCS guideline). It should be taken into account that permeation as a form of diffusion is driven by a concentration gradient, thus the system is not in a state of equilibrium at the beginning of the experiment when the drug is applied. The drug may diffuse slowly or even stop due to interactions, e.g. protein binding inside the skin membrane for a while (non-steady state conditions). This is the so-called lag time (s. figure 10), where

still no drug can be found in the receptor solution. When this time period is passed which may be different and specific for various compounds, an equilibrium is reached and the diffusion (flux) is strictly proportional to the concentration gradient (steady state) [85, 86].

Evaluation of the results of such a permeation study includes besides the P_{app} value and steady state flux (slope) the total mass balance of the tested compound for which the drug residues in the donor, the acceptor and inside the skin have to be quantified [79].

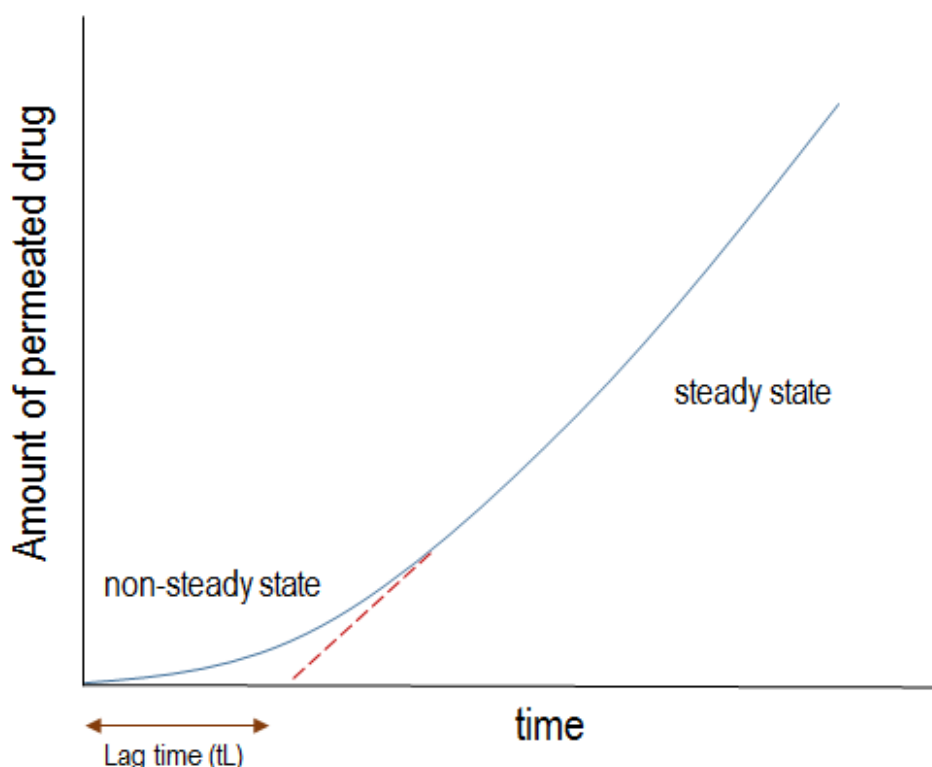


Fig. 10:schematic presentation of a typical permeation curve. t_L =lag time

3.1.2. Penetration/Extraction

If the goal of a study is not the evaluation of a drug's permeation through a membrane (skin) but to see if it is able to penetrate the SC, to which extent if at all, and determine the fractional distribution in the skin layers, a penetration experiment will be conducted. Usually, full thickness skin is either placed in a diffusion cell (as described for skin permeation) in case of a drug solution or in a Petri dish for a semisolid formulation or a patch. The skin specimens are incubated for a defined time at $32 \pm 1^\circ\text{C}$ and remaining drug is removed with cotton balls afterwards. The SC is segmentally removed by tape-stripping. The

remaining skin layers will be segmented as cryo-cuts with a cryomicrotome. The cotton balls, strips and cryo-cuts of each skin specimen are then collected in a specific vessel and filled up with a defined amount of extraction medium which is selected according to the solubility of the used drug and again incubated for a defined time (usually at least 60 min). Afterwards the samples can be analyzed.

Evaluation can focus on recovery (total mass balance) of the drug in the skin fractions, amount of API per skin area of SC and deeper layers or API content in relation to skin volume [84].

3.1.3. Routes of drug delivery through skin

When applied to the skin surface, a drug has three possible ways of penetration:

- Directly across the SC
- Through the sweat ducts
- Through the appendageal route (via hair follicles and sebaceous glands)

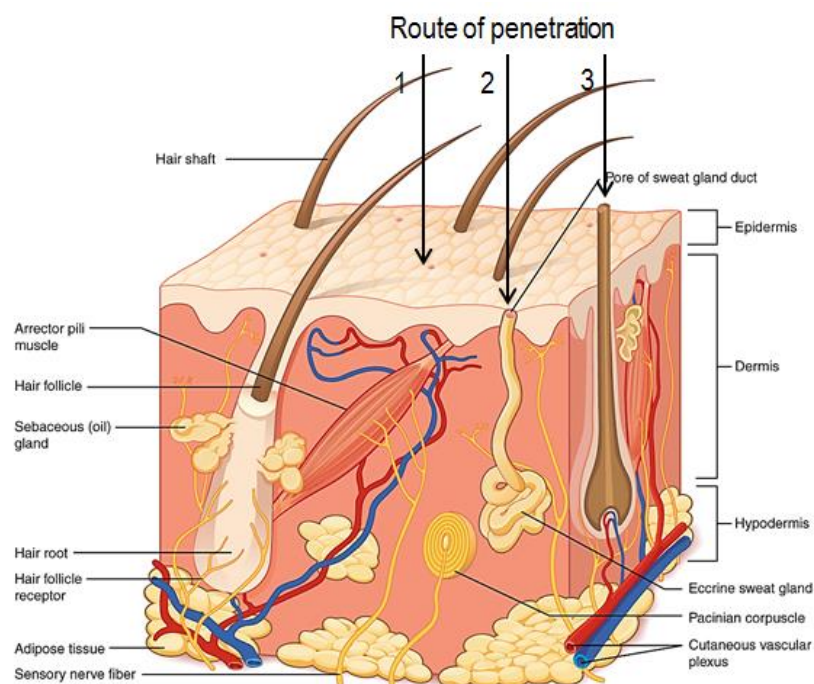


Fig. 11:schematic depiction of skin penetration routes: 1) sweat ducts; 2) across the SC; 3) hair follicles

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It is a general consensus that with a fraction of 0.1 % of the steady state flux the appendageal route contributes only negligibly to permeation although it may have

some involvement in the pre-steady state permeation of large and polar molecules [87, 88]. In fact, the most important route is the diffusion across the SC. Two microroutes can be differentiated here, the intercellular and the transcellular route.

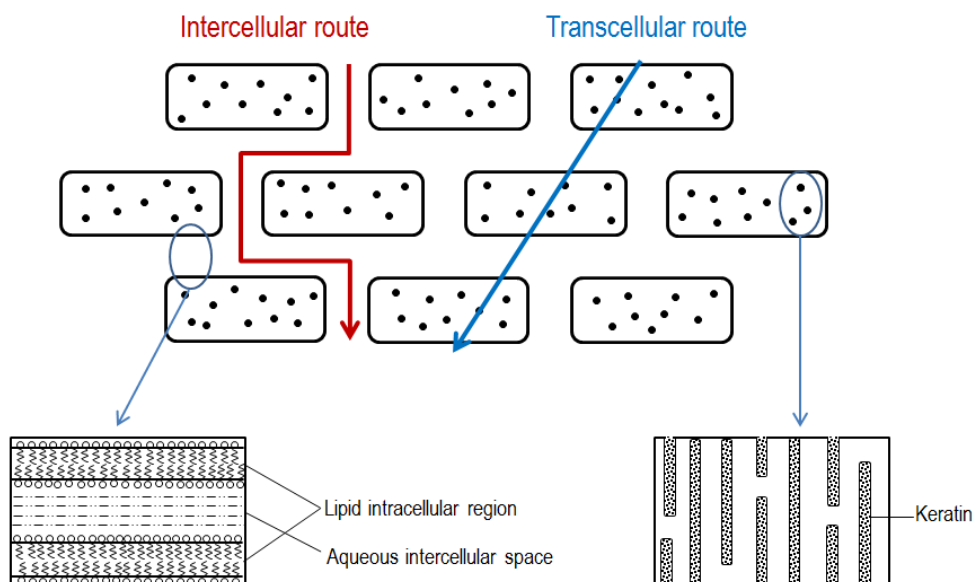


Fig. 12: Schematic depiction of the intercellular and transcellular penetration routes

As previously described, the SC with its “brick and mortar” structure is the main barrier of the skin. Its multiple lipid bilayers formed by long hydrocarbon chains (ceramides, fatty acids etc.) are arranged in lamellar gel, lamellar liquid crystalline and semicrystalline domains with different properties. While it was thought that hydrophilic compounds may favor the transcellular and hydrophobic compounds the intercellular route, this view could not be confirmed and today the intercellular route is regarded as main permeation pathway. Consequently, most methods for enhancing permeation target manipulation or disruption of the intact SC [87, 88].

3.1.4. Enhancement of penetration and permeation

While the first generation of TDD systems mainly concentrated on choice of suitable compounds, different ways of enabling and increasing permeation of a larger variety of APIs were discovered with the second generation.

3.1.4.1. Enhancement based on drug and vehicle properties

Many substances can act as penetration enhances, e.g. water, sulfoxides, fatty acids, esters, alcohols, terpenes, essential oils, azone, epidermal enzymes, polymers and others. Several mechanisms of enhancement can be described which

in many cases are specific for a group of similar drugs.

Disruption of SC lipids

A phase separation of the SC lipid bilayers is induced by lipid disruption which creates transient permeable “pores” in the skin through which the drug can reach the deeper skin layers. Fatty acids, specifically oleic acid, employ this mode of action as well as different terpenes, Dimethyl sulfoxide (DMSO), azone and some essential oils [89, 90]. Extracting lipids from the SC is another mechanism, observed in ethanol and DMSO, hereby creating channels inside the skin [88, 89].

Raising drug solubility

An increase of drug solubility in the skin can be seen with ethanol and propylene glycol (PG) as well as a change of solubility of the SC in the direction towards the employed drug as proven for estradiol, ibuprofen and metronidazole additionally to an unspecific solvent drag effect [89].

Increase in drug partitioning

Ethanol, PG and DMSO also facilitate the partitioning of drugs into the SC. By aforementioned actions, i.e. disorganization of SC lipids, the free volume and the bilayer available for partitioning can be increased and the chemical environment inside the SC is altered [88].

Increase in drug saturation

Higher drug saturation can be obtained by an increase of the drug concentration or its solubility in the vehicle. This results in a higher thermodynamic activity of the drug and so in an increased permeation. It is possible to transiently supersaturate a drug solution to obtain even better permeability but this state is very instable and should be created directly before/during the experiment, e.g by evaporation of a volatile component. Addition of stabilizing agents might be possible [87, 89].

Prodrug

Here, the strategy is to increase the permeation by adding a functional group to the drug which makes its partition coefficient more favorable for skin penetration. After the viable epidermis is reached, the added group is removed by epidermal esterases and the actual “parent” drug is released [87].

Ion-pairs

A modification of the prodrug approach is the usage of ion-pairs. Usually, charged molecules do not easily permeate through skin. By adding an oppositely charged molecule, both charges neutralize each other and the drug can permeate through

the SC. In the aqueous milieu of the viable epidermis the components dissociate and the parent drug is released again [87].

3.1.4.1.1. Nanocarriers

Different structures can be called as nanocarriers, including nanoparticles, liposomes, ethosomes, niosomes, dendrimers and many others, ranging between 1 and 500 nm in scale and thus being small enough to evade the immune system.

Transdermal nanocarrier type	Applications
Liposomes	Can encapsulate both lipophilic and hydrophilic drugs. Positively charged liposomes are used for NDA delivery in gene therapy, antifungal and anticancer applications. Drug examples delivered via liposome TDD: melatonin, ketoprofen, estradiol, indinavir, amphotericin B, methotrexate, lignocaine
Transfersomes	Have been reported to improve in vitro skin delivery of various drugs, penetrate intact skin in vivo and transfer therapeutic amounts of drugs efficiently. Examples: diclofenac, insulin, tetanus toxoid, corticosteroids, DNA, ketoprofen, superoxide dismutase, interleukin-2
Ethosomes	Treatment of atopic dermatitis, Parkinsonian syndrome and dystonia therapy. Examples: tacrolimus, clotrimazole, ketoprofen, testosterone, trihexyphenidyl HCL
Niosomes	Especially potential for cutaneous drug targeting, e.g. feasible cargo carrier for topical minoxidil delivery in hair loss patients. Topical application increases residence time of drugs in SC and epidermis, reducing systemic absorption. Examples: minoxidil, ellagic acid
Dendrimers	Diverse applications such as gene therapy, controlled drug delivery, delivery of contrast agents, catalysts, chemical sensors, cross-linking and light-harvesting agents, also vaccines, antiviral and anticancer therapy. Further act as solubility enhancers, used for biocompatibility and toxicity assessment. Examples: indomethacin, ketoprofen, diflunisal, 5-fluorouracil, peptides
Nanoparticles	Treatment of cancer, diabetes; in case of polymeric nanoparticles also bone healing and vaccination. Examples: minoxidil, triptolide, DNA, dexamethasone phosphate, cyclosporine A, testosterone, caffeine, 5-fluorouracil, chlorhexidine, insulin, celecoxib, coenzyme Q, triclozan, triamcinolone acetonide acetate, flufenamic acid

Fig. 13: summary of nanocarrier applications in TDD, based on [91].

They can greatly vary in structure and chemical composition and be employed for gene therapy, vaccination, antiviral, antifungal and anticancer-treatment as well as for diabetes or atopic dermatitis, see figure 13 [91]. Nanocarriers can improve drug solubility, permeability, stability and bioavailability by influencing cellular uptake based on their shape, size, rigidity and surface charge [91].

Liposomes

Liposomes are the artificial equivalent to naturally occurring vesicles. They are colloidal particles consisting of phospholipids and cholesterol, arranged in concentric layers, encapsulating a drug. They seem to accumulate on the skin surface or in the upper SC layers and fuse there with the endogenous lipids while releasing the drug, an effect that seems to be most significant if the composition of the liposome is similar to those of the skin lipids. Drug delivery was described to be 4 to 5 times higher compared to a simple ointment with the same drug and concentration [87].

Transfersomes

Transfersomes are a specific kind of liposome with phospholipids as their main constituent with added 10-25 % surfactant and 3-10 % ethanol. The function of the surfactant molecules is to act as so-called “edge activators”, making the transfersome ultra-deformable, reportedly 10^5 times more than an ordinary liposome [87, 88]. It is postulated that they can permeate through pores of less than one tenth of their size. Applying the transfersome containing drug solution on the skin under non-occluded conditions, evaporation will cause dehydration of the transfersomes which, in order to maintain their hydration level, will be prompted to follow the hydration gradient from the dry SC to the aqueous viable layers underneath [87, 88]. It was claimed that after 30 min over 50 % of a topical dose of e.g. insulin was delivered by using transfersomes *in vivo* [87].

Ethosomes

Ethosomes are further specialized liposomes, i.e. transfersomes, which can disorganize the SC lipids due to their high ethanol content, hence reaching deeper skin layers and the systematic circulation [87, 88]. They seem to be safe to use and an efficient drug delivery system, especially for lipophilic drugs such as testosterone or propranolol [91].

Niosomes

Another specialized liposome is the niosome, composed of lipids, e.g. cholesterol and nonionic surfactants [87-89]. The aim for niosomes as for ethosomes and

transfersomes was to create liposomes which are less rigid. The addition of surfactant increases their stability. Niosomes are frequently used in cosmetic products, e.g. delivering anti-aging substances but also have therapeutic potential as carrier of antifungal drugs [91].

Dendrimers

Dendrimers are made up of many small molecules, commonly from (poly)amidoamine. They can be fabricated in relatively uniform shape and size and are used to encapsulate instable and hydrophobic components, delivering antiviral, anticancer, antifungal and gene therapies. A disadvantage is their poor biodegradation resulting in cytotoxicity [91].

Nanoparticles

Nanoparticles are another heterogeneous group of nanocarriers. They can be composed of polymers, polysaccharides, lipids, metals and various combinations thereof [92]. They are prepared using techniques such as in situ polymerization, emulsification-polymerization, inverse salting out etc. Next to categorization according to the used material, they can be divided into nanospheres with a solid core and nanocapsules with a hollow core [91].

Polymeric nanoparticles can be tailor-made from degradable or non-degradable polymers, in various sizes and with different surface modifications enhancing their penetration properties. Both they and polysaccharide nanoparticles are especially suitable for TDD [91]. The most common natural polymer for this application is chitosan, a cationic, biodegradable polysaccharide from crustacean shells. Synthetic polymers such as poly-lactic acid (PLA) and poly-glycolic acid (PGA) and their co-polymers are alternatives, all of which are naturally biodegradable [92].

Silver and gold nanoparticles are two examples for metal nanoparticles. While for silver, the active agent seems to be the breakdown product of the particle itself, gold nanoparticles affect drug uptake indirectly via co-delivery. Here, an interaction between the skin barrier and the gold nanoparticle increases skin permeation and facilitates the absorption of the simultaneously administered (protein) drug. Hence, the drug does not need to undergo the complicated loading process into the nanoparticulate system, increasing simplicity and cost-effectiveness [91]. Magnetic nanoparticles are made from iron derivatives and with around 10 nm smaller in size than most other nanoparticles which makes them useful for applications such as cell targeting. They have been proven to

passively diffuse through the SC lipids and hair follicles [92]. Titanium dioxide (TiO₂) and zinc oxide (ZnO) nanoparticles do not penetrate into the SC but form a layer on the skin surface where they reflect and scatter UV rays which accounts for their usage in sunscreens [92].

Solid lipid nanoparticles (SLNs) and nanostructure lipid carriers (NLCs) are also used for TDD [91]. They both act by adhesion, occlusion and skin hydration on the skin barrier, improving drug penetration [92].

Although generally the appendageal route of skin penetration is neglected, nanoparticles have a strong tendency to accumulate in hair follicles. Thus, the penetration of the encapsulated drug can be considerably increased. Moreover, it seems that the drug-loaded particles are stored within the hair follicle while the hair shaft acts as a pump and drives the particles deeper into the follicle reservoir, gradually releasing them into the deeper layers. On the other hand, this mechanism can be also exploited to block the hair follicles with nanoparticles in order to stop or prevent drug uptake via the follicular route [92].

3.1.4.2. Enhancement based on SC modifications

Hydration

Utilization of water is the most common and safest method of enhancing permeability for most substances, whether hydro- or lipophilic. Increasing the water content of the SC can alter the solubility of the drug and thus increase its permeability, in addition the SC structure swells, prompting the lacunae to form a continuous pathway [87]. This increased hydration can be achieved by occlusion of the skin surface with impermeable films (e.g. parafilm), oils, waxes, paraffins and oil/water emulsions. Although not always effective and not for all compounds, a tenfold increase of the diffusion components of alcohols compared to dry skin was observed by Scheuplein and Blank [87].

3.1.4.2.1. Removal of the stratum corneum

As constituent of the main permeation barrier, removing the SC can be an opportunity. This can be achieved by chemical peels, microdermabrasion and laser ablation, but all methods cause damage to the skin and are therefore not suitable for many applications, in addition to high costs in the case of e.g. laser ablation. Tape stripping, however, is occasionally used to remove SC layers

before drug application [88].

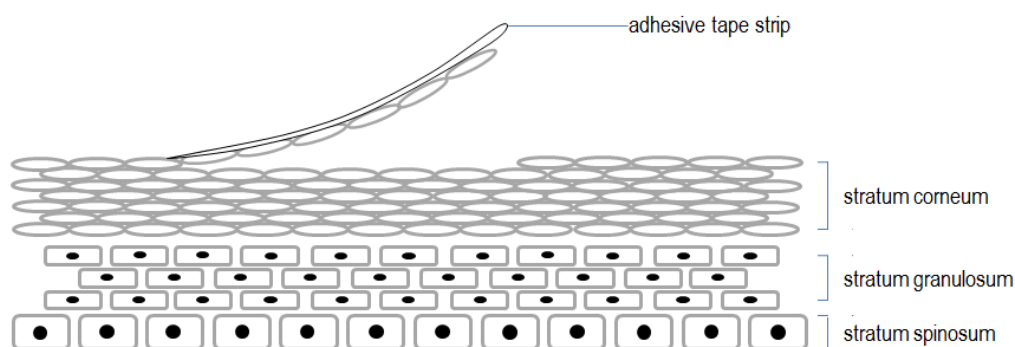


Fig. 14: Schematic drawing of stratum corneum tape stripping, based on [93].

3.1.4.2.2. Bypassing of the stratum corneum

Follicular delivery

The intact SC can be bypassed by using the appendageal route for drug delivery. Offering a higher permeability than the rest of the SC (human skin), a drug can enter the sebaceous glands, the associated hair follicle (s. pilosebaceous unit) or the follicular sheath and directly pass from there through to the dermis. Although the area for diffusion is small, absorption is facilitated by rich blood supply. Sometimes also liposomes target this route. Follicular delivery is especially relevant for large molecules such as DNA which is employed for gene therapy [88]. Due to the higher density of hair follicles compared to human and also porcine skin, in other animal skin, e.g. dogs, this follicular route may be of higher importance than for humans, although the differences in follicular epithelium have to be considered. Follicular delivery offers another advantage for lipophilic drugs as they are dissolved by sebum, thus “trapping” them within the follicular shaft and also providing protection against mechanical removal of the drug. This mechanism is employed by some topical drugs such as fipronil (prevents flea infestation in dogs and cats) [64] [neu].

Microneedles

An array of microneedles is another tool for bypassing the SC barrier. The needles of micron size punch holes into the SC which are large enough for the drug molecules to permeate through but also small enough to circumvent pain or serious damage [94, 95]. The feeling was described as similar to a cat’s tongue in a human study [88]. Several trials reported increased permeability by orders of

magnitude even for large molecules [88, 94]. This makes microneedles especially interesting for cutaneous vaccination (often protein and DNA or parts thereof), targeting Langerhans cells, and gene therapy [94, 95]. Various kinds of microneedles have been developed so far. Solid microneedles from metal or silicon can be used to form holes inside the skin as a pre-treatment before applying the drug in another step. The needles may be also coated with drug to be released when punched inside the skin, or hollow needles may be used to flow the drug into the skin. Dissolvable needles are made from water soluble and biocompatible polymers with the drug embedded inside. When reaching the viable skin layers, the remains of the needle dissolve or degrade in the fluids and release the drug [2]. This approach can be used for a controlled drug release over seconds to several months. Although this technique is very promising, some challenges are still associated with it. The quantity of deliverable drug is rather low for coated microneedles, for hollow needles a drug reservoir attached to it and a pump driving the drug through is required, causing cost and inconvenience. The curing process of the polymers employed for dissolvable microneedles might denature the drug prior to application [2, 88, 94].

3.1.4.2.3. Electric and acoustical methods

Iontophoresis

Iontophoresis is another way to increase transdermal transport for both charged and uncharged molecules [94]. A small current (around 0.5 mA/cm^2) is applied to the skin via a drug containing electrode directly in contact with the skin with a second electrode placed on another body region to complete the circuit [88]. Permeation is enhanced by three mechanisms. Firstly, an electrophoretic driving force, repulsing charged molecules from the electrode and driving them into the skin. Secondly, electro-osmosis, dragging uncharged and large molecules with the induced solvent flow and thirdly permeabilization of the skin through the electric current which seems to act on the follicular pathway at lower voltages and to disrupt the SC lipids at higher voltages [88, 94]. The effect greatly depends on molecular mass, charge, polarity and the formulation and is mostly used for delivery of anti-inflammatory and topical treatment. Concern about possibly irreversible damage to skin and growing hair (induction of follicular route) and difficulties in handling of the apparatus are some considerable disadvantages of this method [88, 94].

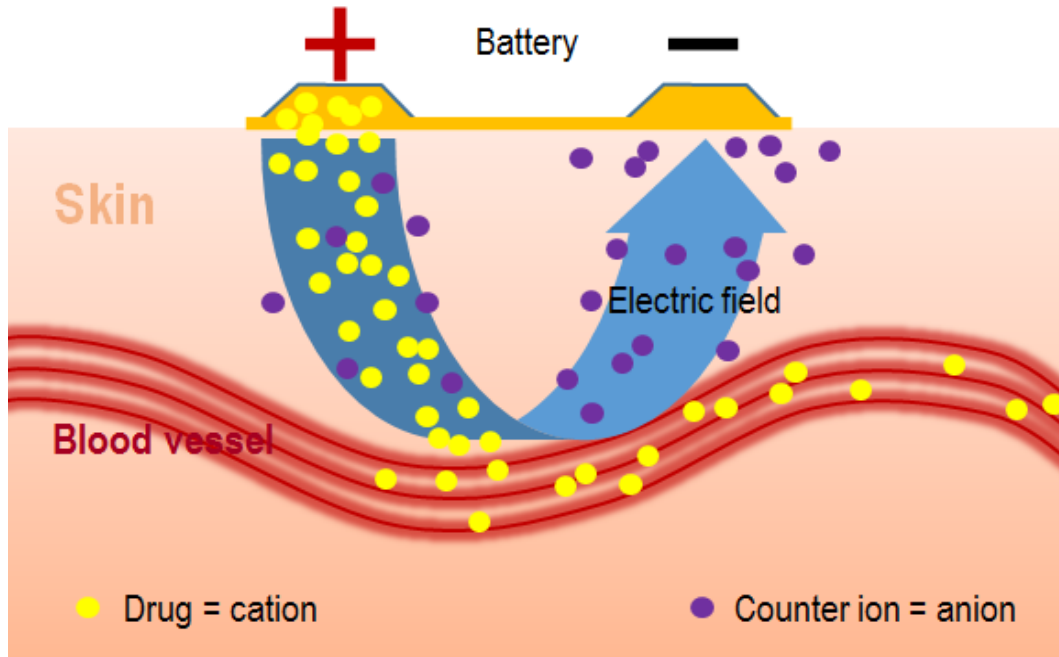


Fig. 15: schematic depiction of iontophoresis.

Electroporation

By electroporation, transient pores in the SC lipids are created by application of short, high voltage pulses (micro to milliseconds, up to 1000 V/cm). In this way, drugs can permeate directly to the viable epidermis. The mechanism employs the aforementioned concepts of iontophoresis, electro-osmosis and the decrease of skin resistance for several orders of magnitude [88, 94].

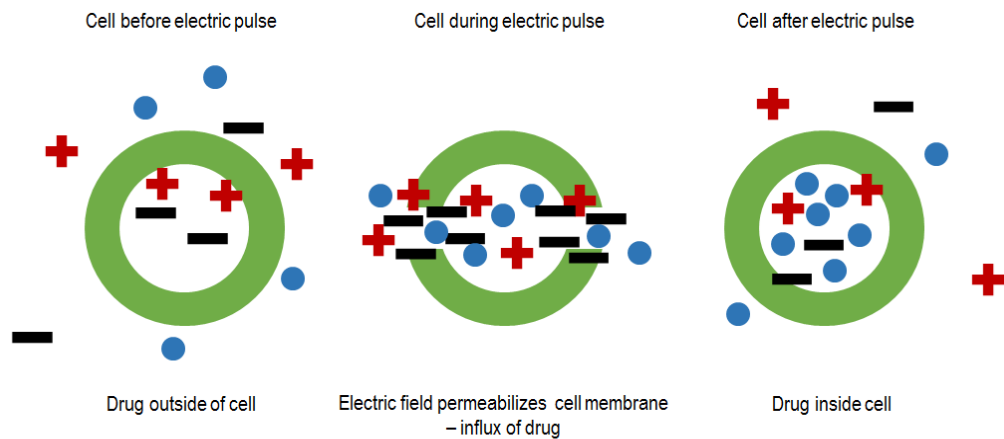


Fig. 16: schematic depiction of electroporation process.

Redrawn based on Neoelegance.co.uk, with friendly permission

Sonophoresis (ultrasound)

Originally used in the context of physiotherapy, sonophoresis has been also discovered for transdermal transport enhancement. The important difference to therapeutic ultrasound is the low frequency (around 20 kHz), which directly targets the skin surface instead of deeper muscular tissues. Low frequency sonophoresis has been proven to increase transdermal transport *in vitro* for various big molecules such as insulin, erythropoietin and interferon. The main mechanism of sonophoresis as permeability enhancer is the disruption of SC lipid packing structure by cavitation. Bubbles are created inside the SC. When they collapse, a shock wave is emitted, leaving free space for the drug to penetrate. Additional mechanisms may involve a heat effect by ultrasound absorption and oscillation of fluids. The effect usually lasts several hours in which the drug may be applied [88, 94].

4. *In vitro* skin models

In order to be able to develop and optimize drug formulations for TDD, both for systemically and topically applied drugs, appropriate skin models are required. Depending on the specific purposes of investigation, a variety of *in vitro* skin models exist up to date. However, most of them represent an isolated stand-alone approach rather than a comparable, standardized and validated procedure that could be widely accepted [14, 15]. Moreover, each of the following skin models is associated with some inherent advantages and disadvantages, making it difficult to define what the ideal skin model should be like. A high similarity to *in vivo* conditions may provide increased predictive potential and reliability but requires more biological complexity with resulting challenges in handling, analytics and costs. However, a lower degree of *in vitro-in vivo* correlation may – possibly, not necessarily – decrease the scientific significance but may constitute the only feasible solution, considering practicability and business economics [14].

4.1. Different state of the art approaches

4.1.1. Artificial *in vitro* skin models

Complementary to *in vitro* skin models based on natural tissues, artificial skin models were designed mostly for initial drug screening. With this approach the selection of drug and formulation candidates could be narrowed down to a few suitable formulations to be further investigated with one of the natural models. This approach aimed to increase the efficiency of formulation optimization as the artificial models had an advantage in simplicity and reproducibility over the natural ones in this early state of drug development [15].

4.1.1.1. Poly(dimethylsiloxane), Silicone

Silicone (PDMS) membranes can be a useful tool for vehicle screening and analysis of their effect on drug transport as the acquired data show a good correlation to *in vivo* data in the literature [15]. Several potential vehicles such as mineral oil, oleic acid, ethanol, glycerin, octanol etc. were studied regarding their effects on the permeation of caffeine and salicylic acid. The model has been improved with PEG 6000 copolymer but is still of limited use for the development of formulations as it has been only tested for drugs in aqueous solution [15].

4.1.1.2. PAMPA

The PAMPA (parallel artificial membrane permeability assay) was developed in 1998 as a high throughput screening assay for the evaluation of transcellular intestinal permeability. A hydrophobic filter coated with phosphatidylcholine served as membrane barrier. This assay was modified in 2009 as skin-PAMPA by introducing a lipid system more closely modeled after *in vivo* conditions. The synthetic ceramides (sic) were used as replacement for the natural ceramides as they are cheaper and more suitable for longtime storage. The permeability of several drugs tested with the skin-PAMPA was correlated with human skin data which resulted in a good correlation of skin-PAMPA and FT skin [15].

4.1.2. Reconstructed human skin equivalents

4.1.2.1. RHE (reconstructed human epidermis)

Different RHE models were introduced since the validation of the two pioneer RHE models EpiSkin® and EpiDerm™ by the ECVAM (European Centre for the Validation of Alternative Methods) in 1998. In 2008, the modified EpiDerm™ SIT and the SkinEthic® Reconstructed Human Epidermis were accepted. These models were mainly designed for the assessment of skin corrosion and skin irritancy or for hazard prediction in the case of the Japanese LabCyte EPI-MODEL 24. However, they were also used for permeation and penetration studies and fast screening of the behavior of nanoparticles [14, 15]. Contrary to the high variation in permeability of human skin, one would expect a significantly higher reproducibility of these RHE models. Although the results of a validation study with different permeation substances showed a decreased variability of the data compared to excised skin, the expectations were still not met [14]. In addition, the barrier properties of the RHE models did not meet the requirements as all of them were significantly more permeable than excised human or porcine skin (10-fold and up to 800-fold increased permeation). Although the absolute permeability was not comparable, the permeation ranking of different substances through the RHE models showed high correlation with human and porcine excised skin, reflecting relative permeation rather well [14, 15].

4.1.2.2. Human skin equivalents

Full thickness reconstructed skin models, so-called human skin equivalents (HSE) are made of a matrix equivalent, usually collagen I, with human fibroblasts for the

dermal part and keratinocytes seeded on top for the epidermal part. To allow epidermal differentiation, they are cultivated at the air liquid interface. Supplements and serum are required for organotypic structuring and survival of fibroblasts [14]. Examples for such HSE models are the GraftSkin[®], EpidermFT[®] and Pheninon[®] models as well as the fibroblast-derived matrix model (FDM), the full-thickness collagen model (FTM), the Leiden epidermal model (LEM) and the full-thickness outgrowth model (FTO) [14, 15]. The barrier properties of these HSEs were more similar to human skin with only a 3-fold to 4-fold higher permeability than excised skin, compared to RHE models (regarding permeation of caffeine, hydrocortisone and tamoxifen). The lipid composition and packing was different to *in vivo* skin, partially due to the relative humidity during cultivation. Although the HSEs were already able to mimic several features of natural human skin, the differences in barrier function and lipid composition limit their suitability for drug development [14].

4.1.2.3. Additional cells

The aforementioned reconstructed skin models are of course a very simplified approach to imitate *in vivo* skin, as many components and cell types still are missing.

Melanocytes: For the investigation of diseases like vitiligo, where melanocytes and skin pigmentation are involved, the establishment of a pigmentation model such as the 3D pigmentation model from Duval *et al.* was necessary to study pathogenesis and response to appropriate drug development.

Langerhans cells: The epithelial dendritic Langerhans cells are one of the most important immune cells of the skin. As such, there were various attempts to incorporate them into HSE models. These models serve to predict allergic reactions, e.g. contact dermatitis.

Endothelial cells and adipocytes: To achieve higher biological complexity and predictive potential, the expansion of HSE models with added blood vessels and subcutis was a logical step. Seeding of human umbilical vein endothelial cells (HUVEC) and fibroblasts onto a scaffold resulted in rudimentary vascularization while for the adipose tissue even a preliminary metabolism could be detected [106].

Hair follicles: While the interfollicular epidermis is an effective barrier for

hydrophilic substances, the hair follicles open a pathway for these drugs. Therefore, the penetration and permeation profile of a HSE model including this pathway may be more close to *in vivo* skin.

Tissue stem cells: Adult mesenchymal stem cells (MSCs) from human bone marrow and adipose-derived MSCs were added to a human 3D skin equivalent. They both promoted the development of a stratified epidermis similar to native skin while the latter ones increased epidermal healing upon laser injury [14].

4.1.3. *Ex vivo*/explant models

Skin for explant models can be taken from healthy donors or donors with a certain disease, depending on the study purpose. They may be used as full-thickness or split-thickness skin, long- or short-term cultivation, with or without serum supplementation and in viable or non-viable conditions, fresh or thawed after freezing [14].

4.1.3.1. Human skin

For the investigation of TDD for human, human skin is obviously the most suitable model. It is usually obtained from surgery or as cadaver skin. Sampling sites are the abdomen, breast, back and leg, rarely also the face [14, 15]. Due to the natural origin of the tissue, the *in vivo* - *in vitro* correlation is generally higher than for other systems. With their complexity, these models can be employed for the study of various biological processes, influences of endogenous and exogenous factors, disease formation and healing attempts, co-cultivation of specific cell types and pharmacological modulation, next to the assessment of penetration and permeation of drugs. For example, full thickness human skin models have been used for the measurement of epithelial outgrowth (keratinocyte migration) and study of populations of dendritic cells. They can also be used for compound screening and, in short-time culture, for skin barrier function reconstruction, the assessment of skin inflammation or as co-culture models with heterologous cell types [14]. Many topical formulations have been tested on human full skin, such as nanoparticles in hydrofluoroalkane foam for the delivery of tocopheryl acetate or the evaluation of celecoxib delivery via ethosomes, liposomes and transfersomes [15]. In addition to its use for drug screening, split-thickness human skin (epidermis) has been used for the study of the transition from the non-proliferative to the proliferative phase during epithelial outgrowth

[14]. In addition to the utilization of healthy skin to study general processes, disease models are also possible, mainly intended for development of topical drugs which should prove their efficacy on skin displaying the typical alterations. The focus here can be on inflammation (psoriasis, atopic dermatitis, vitiligo), trauma (wounds, burns) or neoplastic cells (melanoma, squamous cell carcinoma) [14]. However, in spite of its advantages, human skin comes with certain limitations. Additional to the inherent loss of vascularization and nervous system like other 3D cultures, the high variation of skin permeability between different donors is a problem. The lipid content and composition, skin thickness, hydration etc. are influenced by anatomical site of the explant, sex, age and ethnicity, resulting in variations of up to 45% *in vivo* and up to 66% *in vitro* [15]. Further obstacles are the restricted availability of skin specimens and biological limitations [14, 15].

4.1.3.2. Animal skin

Porcine: After human skin as the “gold standard”, domestic pig skin counts as the second most suitable model [16]. Porcine skin is similar to human skin regarding features such as epidermal thickness, hair follicle density, ratio of dermis to epidermis, SC lipid content, and dermal collagen and elastin fibers. In contrast to human skin, it does not contract after removal, rendering especially porcine ear skin a better model for topical drug delivery via hair follicles than even human skin itself. Skin from other body regions can also be used. A good correlation to human skin could be gained especially for lipophilic substances [15]. Full-thickness porcine ear skin has been employed to study the penetration of resveratrol via ethosomes and transfersomes [96], for the optimization of nanocarriers with minoxidil [97] and for the evaluation of epidermal accumulation of clobetasol from nanoparticles [98], to mention just a few examples. Numerous creams, ointments, emulsions and microparticles were tested using porcine skin. Another model with abdominal porcine skin was used for a delivery system with semisolid vesicles. With split-thickness porcine dorsal skin a microemulsion formulation of testosterone was optimized [99] while newborn pig skin is increasingly considered for topical drug formulations [100]. Porcine ear HSE was employed for the screening of polyamide nanocapsules containing sunscreen filters [101]. Porcine skin shows lower inter-individual variation than human skin and is freely available from local slaughterhouses, without ethical concerns and

almost no cost (ca. 1€/kg skin).

Rodent: In contrast to porcine skin, rodent skin is restricted and its acquisition needs ethical permission. Advantages of rodent skin are their size, easy handling and low costs. However, with their fur coat and resulting density of hair follicles they need to be shaved before usage of skin which may damage the SC barrier. Several nude species are available, such as hairless mice, rats and guinea pigs. Generally, looking at skin morphology, lipid composition, hair follicle density, thickness of SC and water content, rodent skin is more permeable than human skin and not completely suitable as a surrogate. Especially rats and mice yield considerably higher permeation rates. Guinea pigs seem to be the most suitable rodent species. Hairless species may show better result than furry ones.

Others: Other less conventional attempts included primate skin which is not feasible due to ethical restrictions, the perfused bovine udder model (BUS) and shed snake skin. The BUS is very well-correlated and can be used for the comparison of permeation, metabolism and absorption. The udders have to be isolated directly after slaughter and continuously perfused via the pudendal external arteries. The practicability is therefore rather low and the model rather unique and not widely accepted. Another approach is the snake skin model. Shed snake skin has been confirmed to have similarities with the human SC regarding structure, composition, lipid content and water permeability. Moreover, a snake sheds periodically, thus providing a high amount of shed skin of the same individual, eliminating unfavorable variability. Furthermore, the snake does not suffer injury or death to obtain the tissue, no pre-treatment of the shed is necessary and the sheds can be stored at ambient temperature. However, snake skin lacks hair follicles which may influence permeability and makes it unsuitable for drugs penetration via hair follicles. Also there seems to be a high variation between different snake species so that some studies present promising results while others do not. Of course, this list (see figure 17) is by no means complete and further species may be selected [15].

Skin model	Application	Advantages	Disadvantages
Artificial models			
Silicone membrane	TDD	Reproducible, low cost Almost unlimited storage	Non-lipid based, low similarity to SC Non-biological origin
PAMPA	TDD	Reproducible, low cost Prolonged storage	Non-lipid based/synthetic lipids, low similarity to SC, non-biological origin
Reconstructed skin equivalents			
RHE	Skin irritation/corrosion and sensitization, TDD, Phototoxicity, Metabolization,	Consistence in permeability compared to human skin, validated models available, high standardization	More permeable than human skin, questionable barrier function, high cost, low complexity, short-term culture only
RHE + melanocytes	Skin lightening, pigmentation	Standardized model for skin pigmentation	Low relevance for drug development
HSE	Percutaneous absorption, wound healing, bacterial adhesion	Standardized, relevant tool for wound healing process studies	Expensive, only short-term culture
HSE + melanocytes	Expression of melanogenetic proteins, vitiligo pathogenesis	Drug development for vitiligo	Not standardized
HSE + Langerhans cells	Allergen assessment, maturation and migration of Langerhans cells	Immunocompetent model to assess sensitization potential	Not standardized, cell lines used
HSE + endothelial cells and subcutis	Angiostatic therapies Adipose metabolism	Long-term cultivation, assess drug impact on adipose tissue and angiogenesis	No approved standards, technically still immature
HSE + hair follicles	Substance penetration	Influence of hair follicles on substance penetration, no artificial components	Low throughput method Other skin appendages not included
Explants			
Human	Epithelial migration, skin inflammation, compound	The most relevant model	Ethical permission, high variability

Fig. 17: List of different skin models, based on [15] and [14]

	screening, neuronal interaction and outgrowth	All cell types present, drug development	Different anatomical sites, storage, Limited amount of viable skin
Human + hair follicles	Molecular processes of hair follicle formation	Analysis of de novo hair follicle formation, close to physiologic skin environment	Non-uniform developmental stages, no influence of epidermis on hair follicles, not ideal for growth of developed hair follicles
Pig ear	TDD, percutaneous absorption, drug development	Easily obtained Similarity to human skin	Skin thickness influenced by age of animal, hair removal/skin damage, storage
Newborn pig	TDD, percutaneous absorption, drug development	SC thickness similar to human skin	More hair than humans, different anatomical sites, skin thickness differs, storage
Mouse	TDD, percutaneous absorption	Small size, easy handling Hairless species available	Ethical permission, very thin skin highly permeable, dense hair follicles, hair removal/skin damage
Rat	TDD, percutaneous absorption	Small size, easy handling Hairless species available	Ethical permission, thin skin, more permeable than human, high density of hair follicles, hair removal/skin damage
Guinea pig	TDD, percutaneous absorption	Similar permeability to human and pig ear skin, hairless species available	Ethical permission, dense hair follicles, hair removal/skin damage
Rabbit	TDD, percutaneous absorption	Ears waste from slaughter, hairless species available	Ethical permission, dense hair follicles, hair removal/skin damage
Snake shed	TDD, percutaneous absorption	Single animal provides repeated sheds, No harm to the animal, multiple samples from one shed, Storage at room temperature	No hair follicles, different skin metabolism than human, no living epidermis and dermis
BUS	TDD, percutaneous absorption, metabolism	Easily obtained, comparable to living skin Multiple samples from one animal	One donor for testing only one sample, Weaker barrier for some drugs, storage

Fig. 18: List of different skin models, based on [15] and [14]

4.1.4. Skin preparation, handling and storage

Depending on the special purpose, different experimental setups are used. While rodent skin is often used fresh, porcine skin as well as human skin for permeation experiments is mostly stored frozen at -20 °C for six months or more. Although it was long general consensus that permeation of hydrophilic substances does not significantly differ in frozen and fresh human or porcine skin, some studies from 2014 proved the frozen skin to be more permeable than the fresh one. Skin for permeation studies can be prepared as FT skin (often rodents, pig ear or newborn pig which have very thin skin), split thickness skin (human, dermatomed to 200-400µm and pig, dermatomed to 400-700µm), trypsin-isolated SC and HSE. HSE is created by peeling of the epidermis after placing the skin specimen in 60°C hot water for a minimum one minute and is rather unsuitable for hairy skin due to holes from the hair shafts remaining in the dermis. Trypsin isolated SC is produced by leaving the skin in trypsin buffered solution for 24 hours. Both HSE and trypsin-isolated SC were reported to be equivalent in permeation rates [15].

4.1.5. Data correlation

One of the most important points about in biopharmaceutical research is data correlation. As by now, it is mostly not feasible to abstain from *in vitro* models during drug development, it is crucial that data obtained from *ex vivo* studies are correctly correlated to data from *in vivo* studies to allow reasonable comparison. Only then, any significance can be given to *in vitro* results. Another challenge is the huge number of animal species used and different models described in the literature which makes comparisons almost impossible. Slight variations in experimental procedures with the exact same skin model such as the type of diffusion cell, skin temperature, acceptor media, application dose, diffusion area, culture conditions etc. can tremendously affect data [102]. These facts might be some of the reasons why many of all these models are stand-alone approaches rather than defined procedures [14, 15]. As a result, literature data may not be as reliable as they could be, urging researchers to conduct their own studies to answer the same questions already asked before, contributing to the vastness of the field and squandering valuable resources. Therefore, methods to correlate data and validate and standardize experimental procedures are urgently required [102].

4.2. Skin viability

While for permeation and penetration studies generally non-viable skin is used and often no difference is made between fresh and previously frozen skin, this approach is not possible for all applications as for some of them, viable skin is crucial, e.g. epithelial migration, skin inflammation, disease models etc. The necessity for usage of viable skin models imposes a set of difficulties and challenges that non-viable skin does not have. Still, usage of viable skin may have to be considered even for permeation experiments, as several studies have demonstrated the impact of viability and skin metabolism on the results [14, 103, 104]. Kao et al. [103] found metabolic viability of the skin to be a determining factor in skin permeation. In the non-viable skin samples only unchanged parental drug was found in the receptor fluid, whereas with the viable samples parental drugs and a spectrum of metabolites could be detected. Most drugs proved to be inducer, substrate or inhibitor of the involved enzymes of cutaneous metabolism [104]. Inducement by topical benzo[a]pyrene, e.g., led to a two to threefold increase of the permeation of the drug [103]. The conception of skin as a merely passive barrier is hence untenable. Therefore, representative *in vitro* studies should consider both diffusional and metabolic processes to achieve meaningful conclusions [14, 104].

4.2.1. Skin metabolism

Skin metabolism is largely due to enzymes of the cytochrome P450 (CYP) family. They are involved in several homeostatic processes as well as in the metabolism of xenobiotics and can be induced by numerous allergens, toxins and carcinogens to which the skin is exposed. CYP enzymes are located in the endoplasmic reticulum and mitochondria and perform a two-phase oxidative biotransformation, similar to the hepatic biotransformation. A substance may be subjected to degradation or activation, possibly resulting in skin sensitization or carcinogenesis. Normal healthy skin shows expression of CYP1A1, CYP1B1, CYP2B6, CYP2E1 and CYP3A, which could also be detected in organotypic skin models [104]. Upregulation is often associated with (skin) disease and inflammation. CYP are involved in the metabolism of retinoic acid, which is used as drug against psoriasis and several types of acne. CYP enzymes also control the transformation of vitamin D into its active form (1,25-

dihydroxycholecalciferol), its degradation and turnover. Vitamin D is handled as another anti-psoriatic agent. Furthermore, CYP contribute to the biosynthesis of proinflammatory agents which are elevated in patients with psoriasis and other skin diseases. CYP inhibitors such as ketoconazole, clotrimazole, itraconazole etc. are widely successful in dermatophyte therapy. With such an immense involvement in various skin related biochemical processes, this potent enzyme family should not be neglected in drug development [104].

4.2.2. Bridge

When considering the two previous chapters about transdermal drug delivery and *in vitro* skin models, up to 100% of the whole research focuses on human health. Drugs are formulated and optimized for human treatment and skin models of various origins are evaluated based on their ability to mimic *in vivo* human skin. It has been proven that animals suffer from similar or even the same ailments than humans do. Therefore, this thesis is meant to initiate a new approach to the field: The establishment of skin models specifically for the intention of investigating and treating skin diseases of the animal, with particular regard of companion and farm animals.

Prior to that, of course, a lot of basic research has to be conducted to develop a solid foundation, including the establishment of basic models under simple conditions and their validation in terms of accuracy, stability and reproducibility. On this base, further attempts can be built and the models may be adapted, optimized and expanded according to various requirements.

In the following three publications, the establishment of a viable *in vitro* human skin model including the modification of viability measurement procedures, their validation and stability analysis and the transfer and expansion of the human model to a comparative animal model will be elucidated.

The overall aim hereby is, to contribute to the establishment of data sets and validated standards for the veterinary field which are comparable to human medicine and equally qualified.

III. PUBLICATIONS

a. Publications Overview:

1. **Establishment of a novel *in vitro* viable human skin model as a basis for the treatment of human and veterinary chronic skin diseases**
published in Journal of Drug Delivery Science and Technology (JDDST),
Volume 51, June 2019, Pages 695-699
<https://doi.org/10.1016/j.jddst.2019.04.008>
2. **Validation and stability analysis of a modified Lactate dehydrogenase test method to be employed for an *in vitro* viable skin model**
published in Heliyon,
Volume 5, Issue 5, May 2019 e01618,
<https://doi.org/10.1016/j.heliyon.2019.e01618>
3. **Establishment of an *in vitro* model of cultured viable human, porcine and canine skin and comparison of different media supplements**
published in PeerJ,
2019 Oct 3;7:e7811.
doi: 10.7717/peerj.7811. eCollection 2019

b. Publications Detailed:

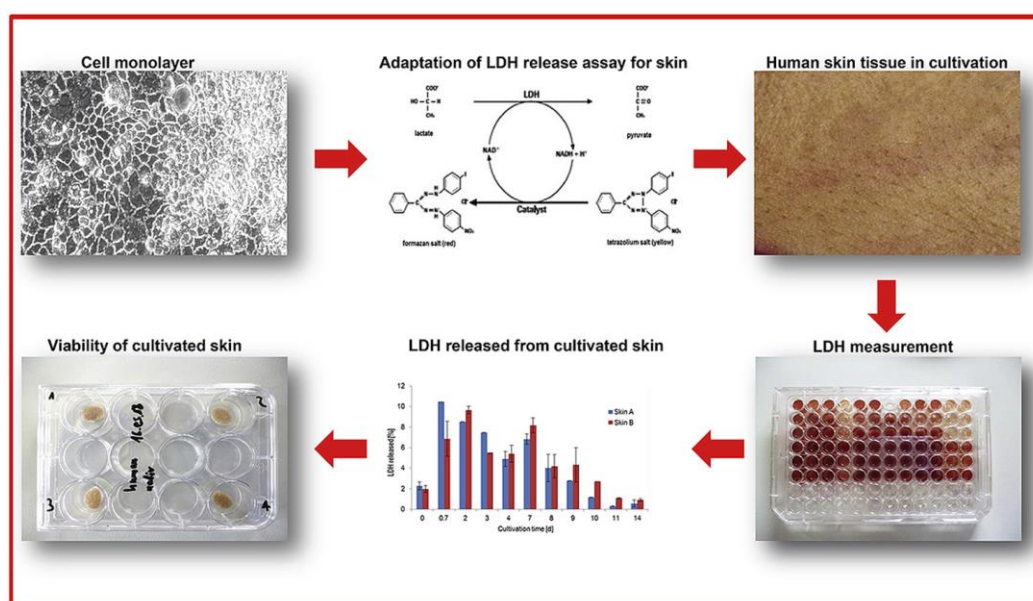
Establishment of a novel *in vitro* viable human skin model as a basis for the treatment of human and veterinary chronic skin diseases

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Abstract

Transdermal drug delivery avoids first-pass metabolism and provides decreased toxicity risk and higher patient compliance compared to gastrointestinal drug administration. Drug development may be significantly shortened by transfer from *in vivo* to *in vitro* testing. The aim of this pilot study was to obtain a viable *in vitro* human skin model, employable for drug development of new formulations for the treatment of cutaneous diseases. Herefore several skin specimens were cultivated under simplest conditions serum-free over 14 days in unsupplemented Dulbecco's modified Eagle's medium at 37 °C/5% CO₂ with the stratum corneum at the air liquid interface. Skin viability was determined with the lactate dehydrogenase (LDH) release assay which is generally designed for cell culture systems. The second aim of this study was therefore the modification of a commercial LDH assay for tissue application. The results of skin cultivation demonstrate, that the skin specimens could be kept at a viability level of $\geq 60\%$ for the first seven cultivation days, followed by a sharp decrease afterwards, being comparable to studies with more complex cultivation conditions. The LDH measurement method was validated, hence it was concluded that the modified assay can be a reliable and useful tool for the viability evaluation of skin tissues.



Keywords: *In vitro*, LDH assay, skin model, skin viability, dermatology

1. Introduction

Chronic inflammatory and infectious skin diseases such as acne vulgaris are a continuous problem [1], [2], [3]. Antibiotic treatment of those disorders is increasingly questioned in the time of rising bacterial resistance [4], [5], [6], [7], [8]. Therefore, new therapeutic options are urgently required. To facilitate and abbreviate the development of new pharmaceuticals and use resources efficiently, *in vitro* models are becoming increasingly popular [9], [10], [11], [12]. Furthermore, in recent years, acceptance for animal testing has markedly decreased [13], [14], [15] and consequently, legislation has issued several rules and guidelines regarding animal testing. The 3R-principle [16], first set up by William Russell and Rex Burch in “Principles of humane experimental technique”, 1959, requests researchers to *Replace* animal testing where possible with another method, *Reduce* the number of animals and/or *Refine* the testing process in such a way that pain and suffering will be decreased to the absolute minimum [16]. With the introduction of new legislation (EU) such as the Directive 2010/63/EU, the establishment of the European Center for the Validation of Alternative Methods (ECVAM) in 1991 and the adoption of the “Declaration of Bologna” in 1999 as well as the proposal of Registration, Evaluation and Authorization of Chemicals (REACH) by the European commission (and similar approaches in the US), *in vitro* research is promoted to be a future standard [16]. Hence, the focus of this study was the development and validation of a novel viable skin model with its main intended application acne vulgaris but also suitable for a wide variety of skin disorders, e.g. also by cutaneous gene therapy [17] and even useful for cosmetic purposes [18], [19], [20]. The use of viable skin is rather challenging compared to non-viable skin, as it has to be obtained directly from surgery, immediately cooled down, transported and prepared for the experiment on the same day and afterwards kept in viable condition for a prolonged period of time [18,21]. Yet, for the investigation of skin disease and as the absorption properties of skin not only depend on the stratum corneum (SC) as the main barrier, but also on the metabolic activity of various cutaneous enzymes and factors [22], a viable skin model has a better predictive potential for *in vivo* conditions. To monitor the skin viability over time, the usage of an appropriate marker is essential. As the viability measurements are repeated several times with the same skin, a non-destructive test method is needed, which

excludes tests such as MTT and WST assay [23,24]. The LDH (lactate dehydrogenase) test is a straightforward alternative although it has to be adapted for the application on tissue instead of cell culture systems [25]. LDH is a ubiquitous soluble intracellular enzyme found in humans and almost every other species [26,27]. It catalyzes the reversible reduction of pyruvate to lactate through conversion of the co-factor NADH to NAD^+ and reverse while the interconversion of NADH and NAD^+ is equimolar to that of pyruvate and lactate [25]. This enzyme can be used as a viability marker as it is only found inside the cells as long as they have an intact cell membrane. If the cells begin to die, LDH is released into the extracellular space, i.e. the culture medium. The amount of LDH in the medium can then be quantified by photometry [25,28,29]. This test could potentially be used also for tissue in the original form, but proper results may not be assured in this way.

Therefore, the main focus of this particular study was on one hand the modification of the LDH release assay for tissue usage including a validation in terms of measurement range and reproducibility and on the other hand the establishment of a viable *in vitro* full-thickness human skin model, cultured serum-free under simple conditions in a basic, unsupplemented cultivation medium over 14 days, monitored with the adapted LDH assay.

2. Materials and methods

2.1. Adaptation of LDH release test for tissue

As an ordinary commercial kit (Cytotoxicity Detection Kit ^{PLUS} from Roche Diagnostics GmbH, Mannheim, Germany) was used for determining LDH release, which is designed for cell culture [30] and not applicable for tissues, the test protocol had to be modified for this purpose. All the measurements were conducted in triplicate.

Sample to reaction mix ratio: With a higher cell density than in monolayer cell cultures, higher LHD values were expected from tissue. In order to determine the needed quantity of each solution for usage on tissue, nicotinamide adenine dinucleotide (NADH) from VWR International GmbH, Darmstadt, Germany was selected as a reference compound. In a first experiment six concentrations of NADH in solution were prepared as calibration standards (named as KLP1 to 6),

ranging from 35.64 µg/mL to 213.87 µg/mL and 50 and 100 µl respectively were pipetted (using piston-stroke pipettes from Eppendorf GmbH, Wesseling, Germany) into a generic 96-well plate (here Nunclon Delta Surface, Thermo Fisher Scientific, Karlsruhe, Germany). After addition of a reaction mixture containing a so-called catalyst and sodium lactate [25], the plate was incubated for 15 min on a horizontal shaker at 150 rpm and either no stop solution, 50 µL of stop solution, or 75 µL of stop solution were added respectively. With different ratios of test solutions in six test runs, the aim was to determine the exact amount needed for each of the components according to the cell abundance in tissue. As the ratio of NADH in the first experiment, called test 1, is twice the amount of the second experiment (test 2), the measured signal after blank subtraction should represent this ratio and be ≤ 0.5 .

Reproducibility: With different tests performed under the same conditions, the reproducibility of the method was assessed.

Stop solution: The necessary amount of stop solution and when it should be added was assessed to ensure, that with the addition of stop solution the reaction is stopped completely but without oversaturation of the reaction. Improper amounts or wrong timing may lead to either under- or overestimated LDH levels and therefore incorrect results. Herefore, a positive control was taken, 50 µL and 75 µL of stop solution added each after 14, 46 and 54 min and measured photometrically with the plate reader.

Establishment of controls: To identify the highest possible LDH release from one skin, a positive control had to be defined for which the skin was mechanically destroyed with an immersion blender. As a comparison, a piece of both fresh skin and of skin stored at -20°C for 3 years was tested. The skins were submerged in a specific amount of DMEM, 12.82 mL/1 g fresh skin and 6.41 mL/1 g frozen skin. After skin destruction the suspension was filtered through a nylon net, the filtrate was centrifuged and the supernatant was aliquoted and stored at 4°C and -20°C for measurement. Positive controls for cultivated skins were obtained by the same procedure. As a negative control, all LDH in the skin was denaturated by heating an aliquot of positive control up to 65°C for 30 min [31]. The negative controls could then be used as blank sample for positive controls, i.e. the measured absorption of the negative control was subtracted from the absorption of positive

controls to remove the influence of turbidity of the medium due to skin matrix. Measurement result of the positive control sample will be considered as 100% of the total LDH amount of this skin specimen.

2.2. Human skin cultivation and viability determination

Several fresh human abdominal skin specimens were obtained from aesthetic surgery (Cabinet Dr. Pierre Sibille, Luxembourg, Luxemburg) from healthy donors with no skin diseases of the same age group. They were processed directly on the day of arrival. The subcutaneous fat was carefully removed with a scalpel and the skin surface rinsed with water. From each skin four full skin punches of 13 mm \varnothing were taken and weighed. The punches were quickly cleaned with 70% ethanol (Waldeck GmbH & Co KG, Münster, Germany) and phosphate buffered saline (PBS) solution (Merck Millipore, Darmstadt, Germany) under sterile conditions (sterile bench laminar airflow, Heto-Holten GmbH, Wettenberg, Germany). Afterwards the punches were placed dermal side down into a 12-well plate (Costar 3513 12-well plates from Corning Life Sciences, Kaiserslautern, Germany) and immersed in 0.7 mL Dulbecco's modified Eagles medium (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) solution per each well, containing 3.7 g/L sodium bicarbonate (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany), glucose (VWR International GmbH, Darmstadt, Germany) and 2.5% gentamycin sulfate (Merck Millipore, Darmstadt, Germany). The SC remained uncovered with medium at the air-liquid interface. The skin specimens were cultivated for 14 days in a sterile incubator (Heracell Incubator, Heraeus, Hanau, Germany) at 37 °C in 5% CO₂/air to keep the pH of the medium at 7.4 [32] which is not only important for the cultured skin but also for the enzymatic reaction employed for measurement [33]. The medium was changed daily, except on weekends. The received medium samples were then measured for the quantification of LDH release into the medium by photometric measurement (microplate reader Wallac 1420 Victor 2) using an absorbance wavelength of 450 nm. Photographs of the skin were taken every working day to monitor the skin's appearance and medium levels. For photometric measurement, the samples were diluted with DMEM solution into the linear range of the LDH calibration curve. Measurements of LDH release were conducted on cultivation days 3, 7, 10 and 14. The resulting amount of LDH inversely correlates with tissue viability. From each fresh skin prepared for cultivation a positive control was made to

compare the measured LDH to the maximum release. For sampling, tubes from Eppendorf and Corning were used.

3. Results

3.1. Adaptation

Sample to reaction mix ratio: In order to determine the required amount of the different components of the LDH assay kit, several pre-tests under different conditions were conducted. In order to evaluate the limiting factor of the reaction, two experiments with NADH concentrations 2:1 were compared (test 1 and test 2). As for all the standard solutions (KLPs) the ratio of test 2/test 1 was <0.5 (Table 1), it was assumed that both reactions were run in excess of reaction mix and 100 μ L of sample + 100 μ L reaction mix were set as amounts for following experiments.

Table 1. Measurement of the absorption of the NADH calibration standards of test 1/2/3 (n = 3).

Sample	Test 1 absorbance w/o blank subtraction [AU]	Test 2 absorbance w/o blank subtraction [AU]	Test 1 absorbance with blank subtraction [AU]	Test 2 absorbance with blank subtraction [AU]	Ratio of absorbance with blank subtraction [AU] test 2 divided by test 1	
KPL1	0.726	0.381	0.671	0.329	0.49	
KPL2	0.629	0.319	0.574	0.267	0.46	
KLP3	0.531	0.260	0.477	0.208	0.44	
KLP4	0.376	0.191	0.322	0.139	0.43	
KLP5	0.243	0.130	0.188	0.078	0.42	
KLP6	0.125	0.075	0.070	0.023	0.33	
Sample	Test 2 absorbance w/o blank subtraction [AU]	Test 3 absorbance w/o blank subtraction [AU]	Test 2 Calculated NADH concentration [μ g mL ⁻¹]	Test 3 Calculated NADH concentration [μ g mL ⁻¹]	Calculated NADH concentration [μ g mL ⁻¹] Mean \pm SD	Calculated NADH concentration RSD [%]
KPL1	0.381	0.374	53.5	53.8	53.6 \pm 0.2	0.39
KPL2	0.319	0.307	44.6	44.0	44.3 \pm 0.4	0.88
KLP3	0.260	0.256	36.1	36.4	36.2 \pm 0.2	0.51
KLP4	0.191	0.187	26.2	26.3	26.2 \pm 0.1	0.29
KLP5	0.130	0.125	17.4	17.1	17.2 \pm 0.2	1.19
KLP6	0.075	0.073	9.4	9.6	9.5 \pm 0.1	1.29

KLP=Calibration standard, AU = Absorbance unit.

Reproducibility: By comparison of test 2 and an additional test 3, both run under

the same conditions, the reproducibility of the developed test method was assessed. The relative standard deviation was $<2\%$ for all the calibration standards.

Stop solution: The evaluation of stop solution efficacy demonstrated a plateau in NADH concentration following the addition of 75 μL stop solution after 54 min of incubation (Fig. 1) rendering that amount sufficient to completely stop the reaction in the tested skin specimen. Thus, 75 μL stop solution were used for the following measurements. Based on the slope of increasing NADH concentration the reaction was not oversaturated.

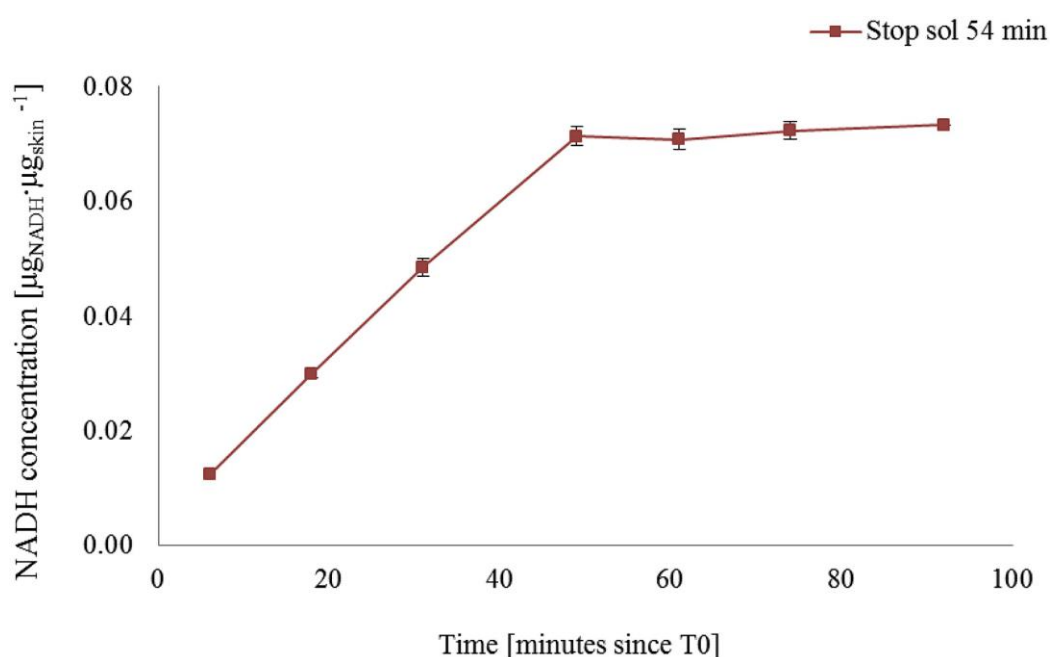


Fig. 1. Measurement of NADH concentrations before and after addition of 75 μL stop solution (after 54 min) over 90 min of incubation ($n = 3$).

Controls: In the positive control of a skin specimen previously stored at -20°C for 3 years, the mean LDH activity was 22156 ± 842 U/g skin. Fresh skin destroyed directly after excision gave a mean LDH activity of 32862 ± 1723 U/g skin. The detected LDH activity in the stored skin demonstrated that LDH was partly preserved in the skin during a storage time of 3 years at -20°C but showed too much loss compared to the fresh skin positive control, to be used for determining tissue viability. The employed destruction method allows differentiating LDH release from skin specimens of different storage times and is suitable to be used to prepare positive controls for this study (see Fig. 2).

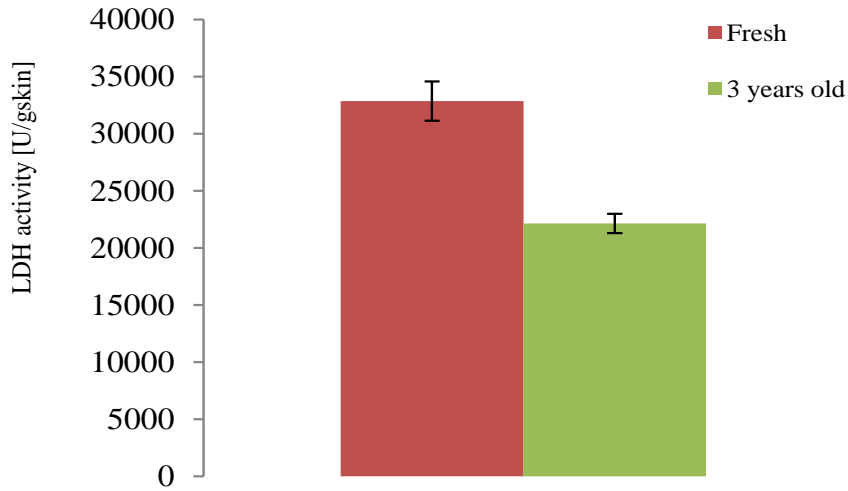


Fig. 2. Comparison of the LDH levels ($n = 3$) of fresh skin and frozen skin to be used for positive control.

3.2. LDH release profile

The two skins displayed an LDH release profile similar to each other (Fig. 3). For both of them, a first peak within 48 h was observed, followed by a plateau phase, a second peak on day 7 and a sharp decrease over the course of the second week. Skin A exhibited its first peak already on day 1 with $10.44 \pm 0.39\%$ LDH release while skin B reached the maximum later, on day 2, with $9.66 \pm 2.39\%$ release. The subsequent plateau phase was clearly shown for skin B but not as demonstrable for skin A. However, in both cases an interim phase between two peaks could be recognized. The second peak on day 7 was with $6.83 \pm 1.61\%$ release for skin A and $8.16 \pm 1.94\%$ for skin B lower than the first one.

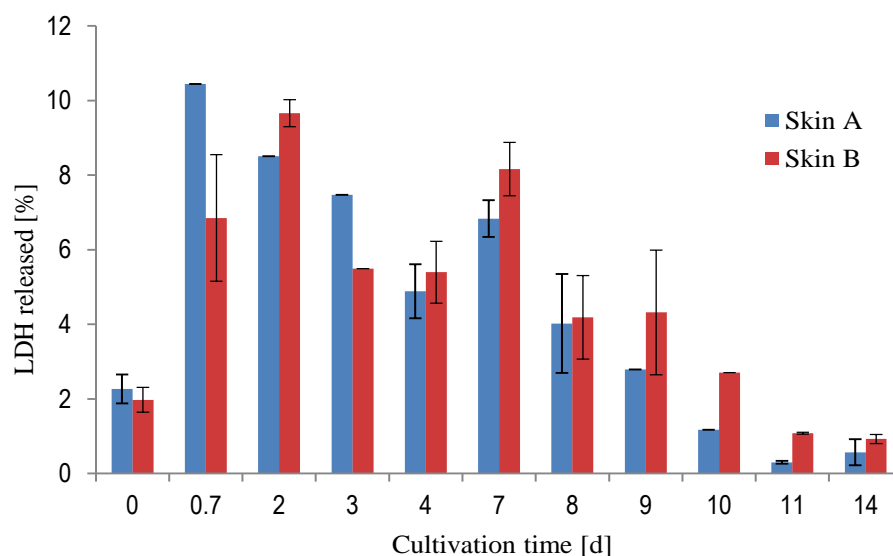


Fig. 3. Course of LDH release in percent of the two 13 mm \varnothing full thickness skin punches (skin A and skin B) over the complete cultivation time of 14 days at 37 °C with DMEM as cultivation medium (n = 3).

3.3. Skin viability

To calculate the viability of the skin tissue from its LDH release, the positive control obtained from the same (fresh) skin was taken as reference. This control displayed a LDH release of 32862 ± 1723 U/g skin. This value represents 100% LDH release of this skin specimen and therefore is equal to 0% skin viability.

The trend of LDH release of two human skin specimens obtained from aesthetic surgery, referred to as skin A and skin B, was monitored over a cultivation time of 14 days. Based on the measurements of LDH activity in AU (Absorbance units), the percentage of LDH release of each skin specimen on every cultivation day was calculated. From the sum of the LDH release percentage of the previous cultivation days, the cumulated LDH release could be calculated (Table 2). From this value, the viability of this skin specimen was calculated (positive control in percent minus cumulated LDH release in percent = skin viability in percent). As after eight days of cultivation $44 \pm 3\%$ LDH (skin A) and $42 \pm 5\%$ (skin B) were released, a residual viability range of 53%–63% (maximum LDH release both A and B 47%, minimum release 37% skin B) for both skin specimens was concluded (Table 2). In this way, it was possible to monitor the gradually decreasing viability of the skin samples over the cultivation time. Both skin specimens were successfully maintained viable for eight days of cultivation under simple

conditions. Starting from day 9, the skin was considered non-vital when $\geq 50\%$ of LDH were released, indicating a skin viability $\leq 50\%$.

Table 2. Mean LDH activity ($n = 3$) as cumulative release for skin A and B over 8 cultivation days.

Sampling time point	T0	T1	T2	T3	T4	T5	T6
Cultivation time [days]	0	0.7	2	3	4	7	8
Skin A							
LDH activity [U/g skin] \pm SD	744 \pm 128	4175 \pm 128	6971 \pm 128	9425 \pm 128	11029 \pm 366	13274 \pm 528	14595 \pm 964
LDH release [%] \pm SD [%]	2 \pm 0.4	13 \pm 0.4	21 \pm 0.4	29 \pm 0.4	34 \pm 1	40 \pm 2	44 \pm 3
Skin B							
LDH activity [U/g skin] \pm SD	649 \pm 109	2900 \pm 666	6074 \pm 785	7877 \pm 785	9649 \pm 1051	12331 \pm 1294	13706 \pm 1663
LDH release [%] \pm SD [%]	2 \pm 0.3	9 \pm 2	18 \pm 2	24 \pm 2	29 \pm 3	38 \pm 4	42 \pm 5

Course of cumulative LDH release over 8 days of cultivation, comparing two different skin specimens (skin A and B). On day 8, skin A has released $44 \pm 3\%$ of its total LDH content and skin B has released $42 \pm 5\%$ of its total LDH, referring to the positive control. A remaining viability of $56 \pm 3\%$ for skin A and $58 \pm 5\%$ for skin B (positive control minus LDH release of the corresponding skin specimen) was calculated. With a viability $\geq 50\%$ for both skin specimens (range from 53 to 63%), both of them were considered still viable on day 8. So both skin specimens were successfully maintained viable for eight days of cultivation.

4. Discussion

In this study, a traditional LDH release assay was adapted for tissue usage by evaluating the sample to reagent ratio, the reproducibility of the method and the efficacy of stop solution. NADH was selected as reference compound. By linkage of this reaction to the reduction of a yellow tetrazolium salt (INT) to a red formazan salt, corresponding to formation of NADH and pyruvate and hence LDH activity, the red formazan salt can be quantified by photometry. The more cells of the tissue are dead or in process of dying, the more LDH is released through damaged cell membranes and the more formazan salt is detected [25]. For the evaluation of the sample to reagent ratio it was crucial to always ensure in-

excess availability of reaction mixture because otherwise with one of its constituents, e.g. catalyst or lactate (substrate) being the limiting factor of the reaction, a correct evaluation of LDH would be impossible. To obtain the best efficacy of stop solution, different amounts and application times were investigated as too early application might interrupt the reaction and lead to underestimated LDH results, late application to an overestimation or improper readings, as the reaction continues as long as enough of the needed reagents are available, causing an oversaturation and making measurements unreliable. The LDH release profile of both investigated skin specimen over the cultivation time of two weeks, displayed the characteristic shape described in relevant literature [34,35] where two distinct peaks separated by a plateau were observed. However, in the described former studies the culture medium and conditions were more complex than in the current study where the aim was to keep the conditions as simple and cost efficient as possible. Human abdominal skin tissue was cultivated with 10% serum and hormonal supplementation for up to 216 h at 32 °C by Reus [35] while in the present study a serum-free culture of abdominal skin in basic DMEM without supplementation at 37 °C was conducted. Human scalp skin was cultivated under serum-free conditions with hormonal supplementation over 22 days at 37 °C by Lu [34] with the main distinction to the current study being the use of scalp skin in order to investigate hair follicle growth, different cultivation medium, treatment of the skin and significantly smaller size of skin specimens (2 mm biopsy).

In both studies the first peak was observed after 24 h, while in the present study one skin's first peak was consistent with these findings and the other skin displayed the first peak on day 2. The second peak was described on day 8 by Reus [35] and on day 16 by Lu [34], respectively. Under the culture conditions of the current study the second peak was reached on day 7. Therefore, culture conditions are the main factor influencing the skin viability. The addition of selected media supplements may increase the lifetime of the tissue in culture.

The first peak generally originates from cell stress and cell death [29], mainly direct necrosis, corresponding to a cellular-trauma induced by a physical factor as the excision of the tissue, but also due to environmental changes (culture conditions). Afterwards the tissue gets adapted to its new environment and less LDH is released. The second peak may be linked to the time point of maximum

apoptosis rate. After this peak, the LDH release dropped down rapidly along with the skin's viability, altering the correlation pattern between low LDH release and high tissue viability. As many skin cell membranes were already opened and the cells dead or in process of dying, most of the contained LDH was already released before, so no more LDH was left to be released further on. The course of skin viability over the two weeks of cultivation was regularly assessed with the LDH release assay, which demonstrates a very close correlation between LDH release and cell death [29]. A cumulative LDH release of approximately 45% until day 8 was observed, from which a residual skin viability of around 55% was concluded, followed by a sharp decrease thereafter. As the viability limit was set to 50% prior to the study, the skin was considered non-vital from day 8 which was further supported by visual signs of degradation, in accordance with the literature [36].

5. Conclusion

The establishment of this viable skin model is a first important step towards a more modern method of drug screening and testing *in vitro*, and evaluating human and animal disease. In order to improve the dermatological health of humans, companion and livestock animals, this may lead to new insights about the underlying disease processes of various skin disorders, helping to find new therapeutic solutions.

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Declarations of interest

None.

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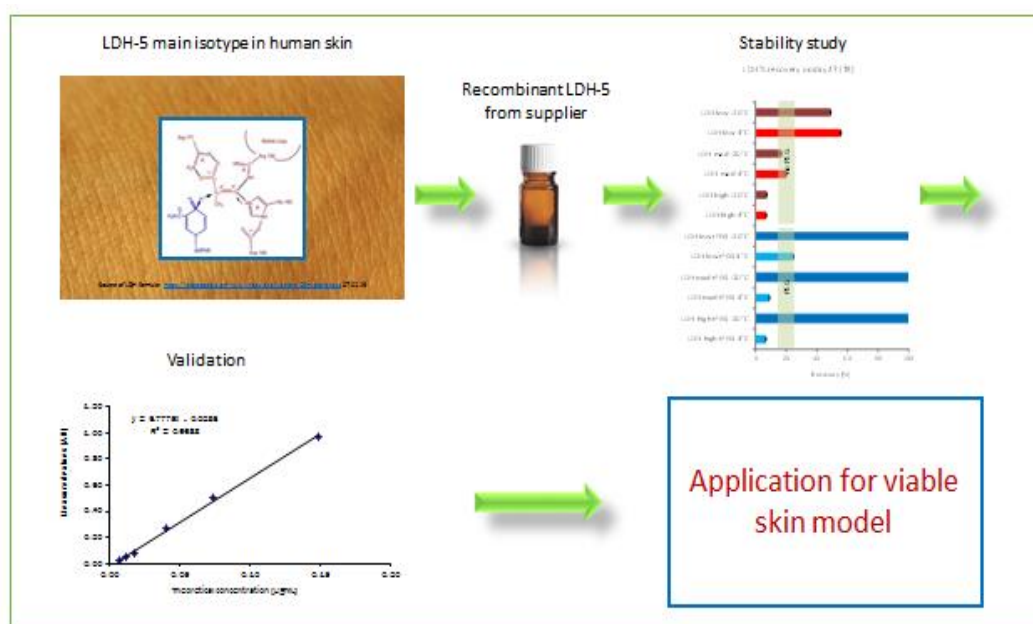
Validation and stability analysis of a modified Lactate dehydrogenase (LDH) test method to be employed for an *in vitro* viable skin model

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Abstract

In view of increasing numbers of dermatological disorders, transdermal drug delivery along with *in vitro* research is becoming increasingly popular. Herefore, qualified *in vitro* skin models are required. The objective of this study was the optimization and validation of a modified lactate dehydrogenase (LDH) release assay during the establishment of an *in vitro* viable human skin model, employable for a variety of skin associated disorders. Firstly, the most suitable LDH isoform for the study was determined. Subsequently, a stability study was conducted to investigate the best storage conditions of the LDH enzyme. Finally, the test system was validated in terms of linear range, range limits and system suitability. The results indicate LDH-5 as most suitable isoform due to its predominance in skin. The stability samples stored at -20°C in the presence of polyethylene glycol (PEG) as cryoprotector displayed the targeted recovery of $100\% \pm 15\%$ until the end of the four-week study in contrast to other investigated conditions. A six-point calibration without PEG and a seven-point calibration with PEG including evaluation of system suitability and quantification limits were established with both correlation coefficients r^2 above 0.99 and all deviations below 15%. Concluding from those results, this method can be considered valid and useful for its employment in viability determination of viable *in vitro* skin models.



Keywords: Biochemistry; Biotechnology

1. Introduction

Dermal health is one of the greatest concerns in both the human and the veterinary field, as skin related disorders, especially allergies, but also infections are rising worldwide [1, 2, 3]. Increasing resistances can make conventional therapy very time consuming and often ineffective [4, 5, 6]. Therefore, new therapeutic options are required.

Because of the complicated and expensive pharmacological development process, *in vitro* research became more and more popular over the last years [7, 8]. *In vitro* research also addresses the controversial issue of animal testing. Social acceptance of animal experimentation declined over the last years due to ethical reasons [9, 10] and resulted in new scientific principles [11] as well as international legislation strengthening animal rights (e.g. Declaration of Bologna in 1999 etc.). Furthermore, the outcome of these tests may, in many cases, not be transferable to humans [12, 13]. Thus, *in vitro* research was gradually becoming more important, as it can be a solution to many of those problems, leading to the development of different skin models. The main aim of this research was the establishment of an *in vitro* viable skin model for the investigation of cutaneous diseases and as basis for the development of new drug formulations [14]. It may also be suitable for other applications, e.g. gene therapy [15]. While for most of those possible applications, the evaluation of percutaneous absorption with non-viable skin may be sufficient, for the investigation of skin diseases and inflammatory processes, a more complex approach is necessary. Usage of viable skin can be challenging, as it has to be maintained viable for a certain time [14]. However, considerable metabolic activity was shown in viable skin [16]. These metabolic enzymes strongly influence the uptake and efficacy of drugs and lead to a better simulation of *in vivo* conditions [16].

Therefore, an *in vitro* human skin model was established. Herefore, human (or animal) full skin is freshly obtained from cosmetic surgery. The still viable tissue is cleaned and cut into several specimen. These are cultivated at 37 °C/5% CO₂ with the stratum corneum at the air-liquid-interface for two weeks. Every day, the cultivation medium is changed and samples taken which are then analysed with a viability marker. In this way, as first step, the development of skin viability under different conditions can be monitored and optimized. A second step could involve

the application of different (e.g. anti-inflammatory) nanoformulations and the evaluation of their effects on the tissue.

For the determination of skin viability, a suitable method had to be chosen. Measurement of TEWL (transepidermal water loss) showed a low correlation to skin barrier integrity *in vitro* and was described as unreliable in the literature [17]. As the viability of the same skin tissue had to be assessed continuously at different time points, a non-destructive method was required which excludes the popular WST or MTT tests acting destructive on tissue [18, 19]. Hence, the non-destructive LDH release assay was selected as viability marker (Roche, 2016). This assay was previously modified for tissue usage instead of cell culture systems. It is also simple to use, provides fast results and does not need expensive equipment. In order to be able to use this assay in the above-mentioned way, and to ensure reliable results, this test method has to be validated. Therefore, one aim of this particular study was to conduct a partial validation, including the following parameters: Linear range, range limits and system suitability. A stability trial was another part of this study, as the issue of LDH stability in various conditions is controversially discussed in the literature [20, 21, 22, 23] and no conclusion could be drawn. A literature review about nomenclature and specific properties of LDH isotypes was also included in this study.

2. Materials & methods

2.1. Basic research on LDH nomenclature and isotype distribution

In order to be able to perform any study with an enzyme, information about its specific properties has to be collected, i.e. temperature and pH optimum [24], substrates, storage conditions etc. The potential existence of several serotypes should be clarified as well as their major differences in catalytic activity. Therefore, intensive literature research was conducted about LDH isoenzymes and their features.

2.2. Determination of LDH stability in different storage conditions

For this experiment, recombinant LDH from rabbit muscle with a specific activity of 844 U/mg protein was diluted in Dulbecco's modified Eagle's medium (DMEM) solution (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany). Sodium bicarbonate (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany),

glucose (VWR International GmbH, Darmstadt, Germany) and gentamycin sulfate (Merck Millipore, Darmstadt, Germany) were added to the solution. PEG 400 (VWR International GmbH, Darmstadt, Germany) was selected as cryoprotector [25]. For LDH measurement, the Cytotoxicity Detection Kit ^{PLUS} (Roche Diagnostics GmbH, Mannheim, Germany) was used. LDH catalyzes the conversion of pyruvate to lactate and reverse by reducing/oxidizing the co-factor NADH/NAD⁺. This enzymatic reaction indirectly produces a red formazan salt, which can be quantified by photometry [26].

The stability study was conducted over four weeks with measurement time points directly after preparation (T0) and on days 1, 3, 6, 8, 10, 14, 20 and 27 (Table 1) with a high (0.438 U/mL), medium (0.135 U/mL) and low (0.034 U/mL) concentration. Each of them were measured in six-fold determination, with PEG and without, and stored at 4 °C and –20 °C. Two stock solutions were prepared, from which the samples were diluted to the corresponding concentration levels. Fifteen percent of PEG were added to one of the stock solutions. The same amount of DMEM was added to the stock solution without PEG to exclude any measurement interferences due to concentration and volume. Blank samples (only medium) were carried with and without PEG to evaluate the influence of PEG on the absorbance. For the measurement, 100 µL of sample per well were pipetted into a generic 96-well plate (Nuncclon Delta Surface from Thermo Fisher Scientific, Karlsruhe, Germany), using piston-stroke pipettes (Eppendorf GmbH, Wesseling, Germany). 100 µL of reaction mixture (kit) were added and the plate incubated for 30 min. at 150 rpm on a horizontal shaker. After adding 75 µL stop solution (normally the enzymatic reaction would continue as long as substrate is available. To ensure that each plate is incubated exactly 30 min, the reaction is terminated with the stop solution), the plate was gently shaken by hand for ca. 10 seconds and then measured by photometry in a plate reader (Microplate reader Wallac 1420 Victor 2). All the numerical results (expressed as LDH activity in absorbance units) were divided by 2.75 during evaluation. This dilution factor is caused by the addition of reaction mix and stop solution (100 µL sample + 100 µL reaction mix + 75 µL stop solution = 275 µL. Hence, the sample is diluted in the ratio 1:2.75 which has to be considered in the evaluation, in order not to underestimate the measured activity). Tubes from Eppendorf and Corning (Corning Life Sciences, Kaiserslautern, Germany) were used for sampling and

measurement. All the measurements were performed in triplicate.

Table 1. Timetable and conditions for stability study.

Stability	Week 1				Week 2		Week 3	Week 4	
TP/timeline	T0/day	T1/day	T2/day	T3/day	T4/day	T5/day1	T6/day1	T7/day2	T8/day2
	0	1	3	6	8	0	4	0	7
Refrigerato r + PEG	RT	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C
Freezer + PEG	RT	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C
Refrigerato r − PEG	RT	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C	4 °C
Freezer − PEG	RT	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C	−20 °C

TP = time point, RT = room temperature (22–25 °C).

2.3. Method validation

To ensure the accuracy and reproducibility of the developed test method, the modified LDH release test was subjected to a partial validation including linearity (calibration curve), system suitability test (SST) and measurement range limits (ULOQ = upper limit of quantification, LLOQ = lower limit of quantification), with acceptance criteria loosely based on some of the Food and Drug Administration guideline for bio-analytics [27].

The establishment of calibration curves for enzyme assays can be challenging, therefore a three-step approach was carried out as following. For the first preliminary experiments a four-point (partial) linearity was sufficient while in another step for the planned main study a six-point (full) linearity was targeted. As in the course of the stability study the usage of PEG was evaluated, a third linearity containing PEG was needed, targeting seven points. For all of them the preparation principle was the same, only for the last one 15 % of PEG were added to the stock solution.

Known concentrations of the re-suspended LDH enzyme were prepared as calibration standards and measured according to the same method described in the stability section. For each measurement series an array of six blanks was carried, and mean blank absorbance subtracted from the received absorbance values of the

standards. The LLOQ was calculated based on the mean blank absorbance plus standard deviation of the blanks multiplied by 3 in accordance with the guideline. The highest standard was defined as ULOQ. The SST was performed using an LDH activity level in a medium-high range of the calibration in six fold determination. Based on the guideline, $\leq 15\%$ RSD (relative standard deviation) for the calculated activity of LDH was set as acceptance criterion for each validation parameter except LLOQ, where $\leq 20\%$ marked the limit of acceptance. All the measurements were performed in triplicate. The mean of the three values was reported.

Data evaluation: The obtained absorbance values with blank subtraction were plotted against the corresponding theoretical concentrations. The coefficient of correlation (r^2) and the back-calculated calibration standard activities were reported.

3. Results

3.1. Basic research on LDH nomenclature and isotype distribution

The different isoforms of LDH enzyme are listed in Table 2. Five naming systems are used in parallel. One refers to the different isotypes as LDH-1/2/3/4/5. The others depend on the prevalence and distribution of LDH subunits H and M with $A = M$ and $B = H$. H₄/H₃M/H₂M₂/M₄ corresponds to B₄/B₃A/B₂A₂/B₁A₃/A₄, while each of them can be also written as HHHH, HHHM, HHMM, or BBBB, BBBA etc.

Table 2. Description and comparison of LDH nomenclature.

LDH Isotypes	Isotype 1	Isotype 2	Isotype 3	Isotype 4	Isotype 5
	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
	H ₄	H ₃ M	H ₂ M ₂	HM ₃	M ₄
Synonym names	HHHH	HHHM	HHMM	HMMM	MMMM
	B ₄	B ₃ A	B ₂ A ₂	B ₁ A ₃	A ₄
	BBBB	BBBA	BBAA	BAAA	AAAA

There are five main isotypes of the LDH enzyme, composed of two tetramere subunits (Fig. 1) and commonly named as LDH-1 to LDH-5. They differ in catalytic activity, mostly depending on the amount of each subunit. There are two

“pure” forms, HHHH (H4) and MMMM (M4) and three hybrids HHHM (H3M), HHMM (H2M2), HMMM (HM3). M-rich isoforms have a higher activity in the presence of high pyruvate concentration, hence being predominant in tissues with considerable anaerobic metabolism, e.g. skeletal muscle or liver (reduction of pyruvate). The activity of H-rich isoforms is inhibited by high pyruvate concentrations and they are predominant in tissues with aerobic metabolism, e.g. heart (oxidation of pyruvate) [28, 29]. Following the same principle, H units can also be termed B units and M units as A [30].

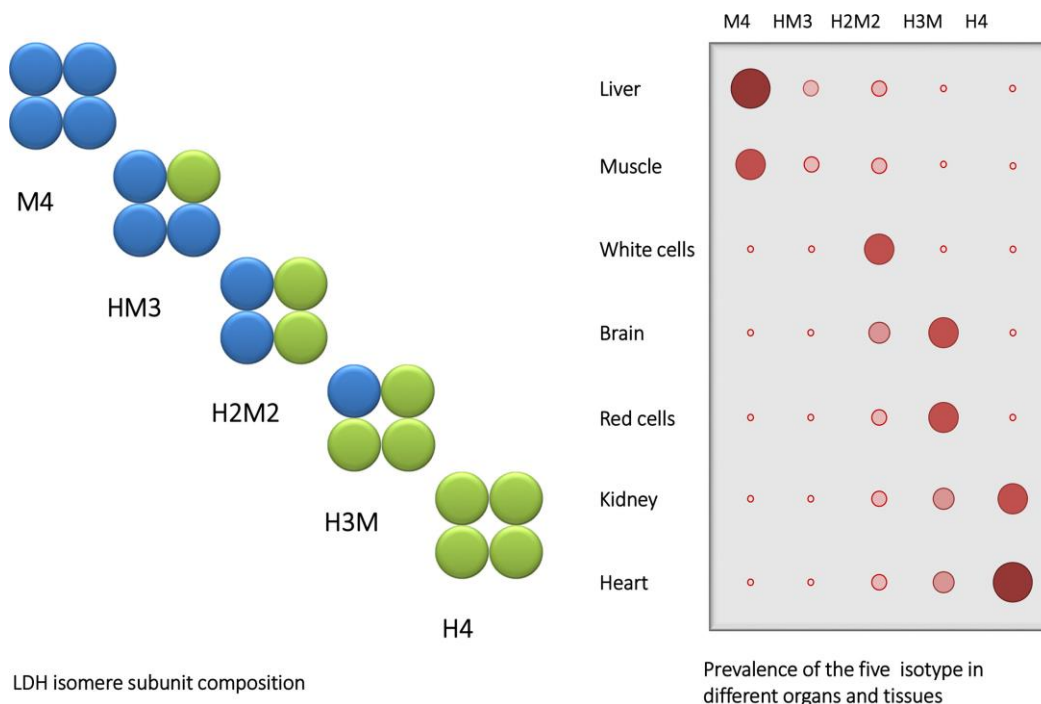


Fig. 1. LDH tetramers and isotype distribution in the human body.

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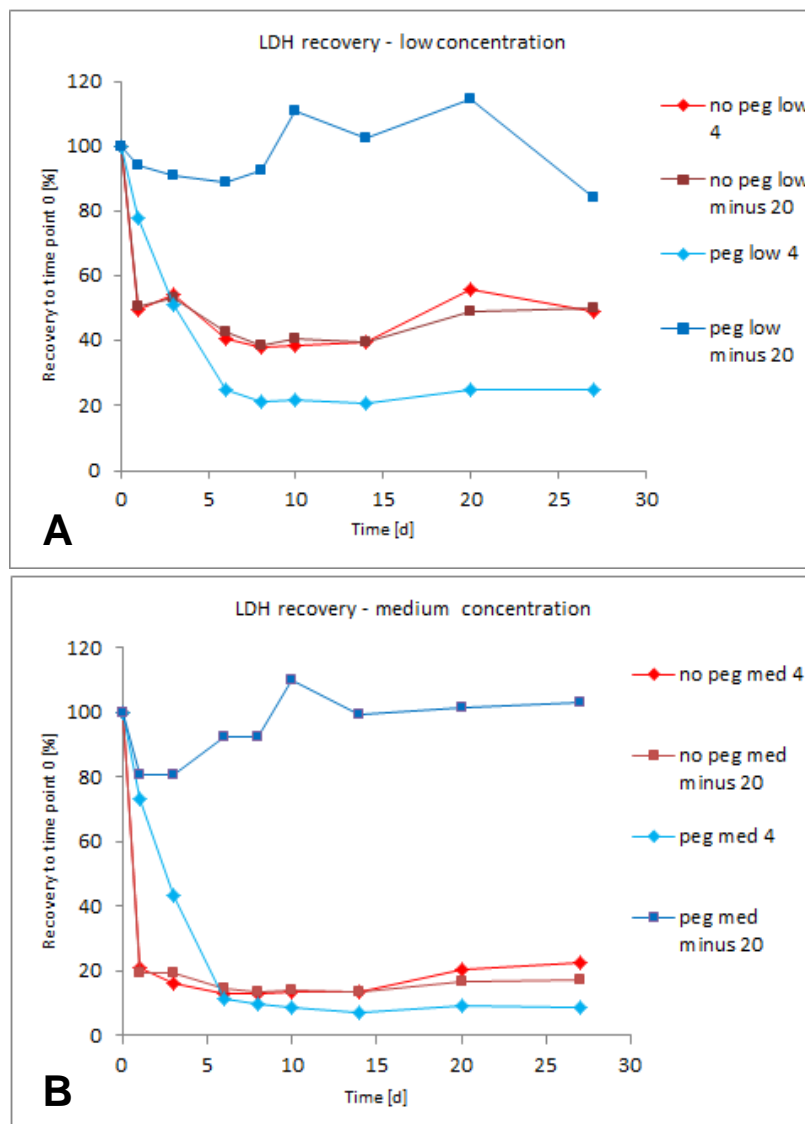
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The predominant LDH form in skin is LDH-5, followed by LDH-4 [30, 31] and LDH-3. Isotypes LDH-1 and LDH-2 cannot be found in the epidermis and the percentage of subunit M and the ratio of LDH-5 to LDH-4 is significantly higher than in the dermis, where all five isoenzymes can be detected [32]. It was essential to compare the main LDH isoform of the tested LDH enzyme with the main isoform of the used tissue, as, if they do not match, the obtained results would not be representative. LDH-5 is the main LDH type in the skin. In the recombinant enzyme LDH-5 was also the main isotype, in a similar ratio (based on the information obtained from supplier). Therefore, the results of all the experiments with the recombinant LDH, especially concerning storage conditions, can be

regarded as comparable to skin and therefore valid.

3.2. Stability of LDH

The outcome of the stability test is summarized in Figs. 2 and 3. Fig. 2 shows for all three concentrations that only storage with PEG at -20°C kept the recovery above 85% until the end of the four weeks. The samples without PEG at both temperatures (4°C , -20°C) show a sharp drop of recovery to around 50% (low samples) or 20% (medium and high samples) on the second measurement time point after 24 h of storage. From there, the recovery either decreased further to around 7 % (high samples) or stayed in this range (low and medium samples). The samples with PEG stored at 4°C also exhibited a marked decrease, ranging from approx. 25% recovery (low samples) to 12% (high samples). This decrease was less pronounced than for no-PEG samples and their level was reached after 6 days of storage on the fourth sampling time point.



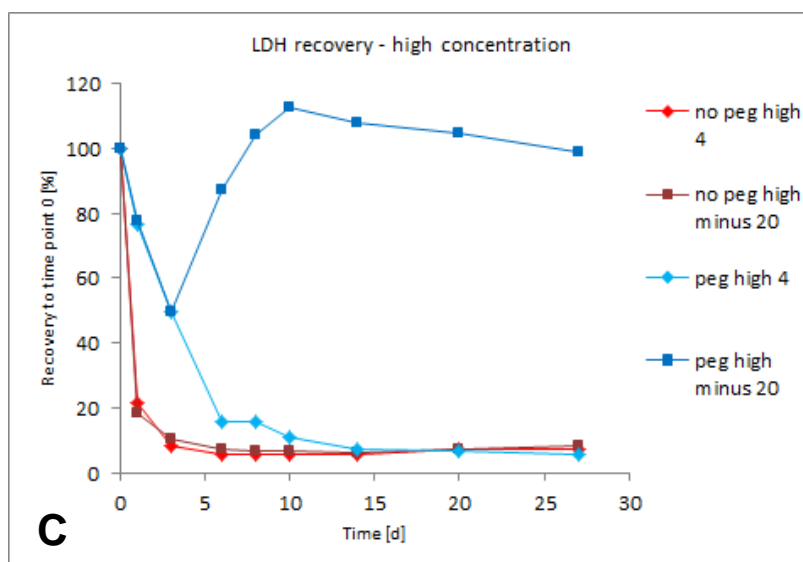


Fig. 2. Display of LDH recovery [%] over the course of 27 days. A: low concentration (0.034 U/mL), B: medium concentration (0.135 U/mL), C: high concentration (0.438 U/mL). All samples measured once directly after preparation (=T0), and on day 1/3/6/8/10/14/20/27 (n=3).

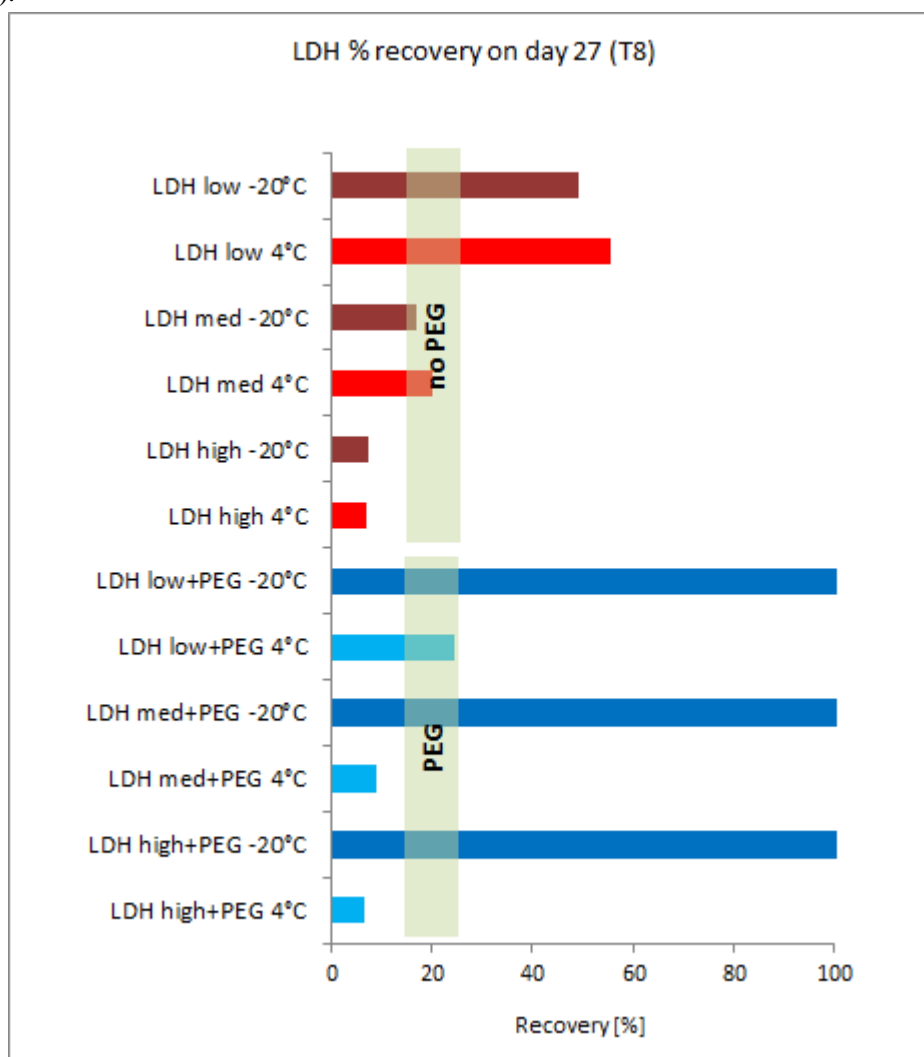


Fig. 3. Plot of measurement results on day 27 for all the 12 investigated storage conditions, showing the difference between PEG samples and no-PEG samples as well as the difference between 4 °C and -20 °C and the concentration levels low, medium and high (n = 3).

The twelve possible conditions resulting from the experimental setting were then plotted into one graph to facilitate the evaluation of the different influences and detect potential codependencies of certain factors, as seen in Fig. 3 where the stability of LDH at the end point of the study (day 27) is shown. There, the samples with low concentration and without PEG displayed a recovery of $48.95\% \pm 0.001\%$ ($4\text{ }^{\circ}\text{C}$) and $50.04\% \pm 0.001\%$ ($-20\text{ }^{\circ}\text{C}$). The medium samples without PEG maintained a recovery of $22.67\% \pm 0.02\%$ ($4\text{ }^{\circ}\text{C}$) and $17.29\% \pm 0.01\%$ ($-20\text{ }^{\circ}\text{C}$) while the high samples showed recoveries of $6.99\% \pm 0.02\%$ ($4\text{ }^{\circ}\text{C}$) and $8.01\% \pm 0.001\%$ ($-20\text{ }^{\circ}\text{C}$).

All the samples with PEG showed more differences between storage temperatures. At $4\text{ }^{\circ}\text{C}$ the PEG samples range from $5.76\% \pm 0.002\%$ (high) to $8.66\% \pm 0.001\%$ (medium) and $24.6\% \pm 0.001\%$ (low). At $-20\text{ }^{\circ}\text{C}$ the recovery of PEG samples for all three concentration levels was finally within the favorable recovery range of $100\% \pm 15\%$ ($85.1\% \pm 0.02\%$ low, $102.97\% \pm 0.03\%$ medium, $98.70\% \pm 0.22\%$ high concentration).

Residual standard deviation (RSD) of all the stability results was $<15\%$.

3.3. Method validation

For the 6-point calibration without PEG (Table 3) a linear range from 0.020 U/mL to 0.409 U/mL (concentration in tubes, before dilution for measurement) was established with six calibration standards, each measured in triplicate. The determination coefficient r^2 was 0.9983 and hence higher than the acceptance value of ≥ 0.99 and the deviations (RSD) ranging from 8.19% to -14.92% within the acceptance limits of FDA-guideline (deviations $\leq 15\%$, for LLOQ $\leq 20\%$). The ULOQ was set to 0.974 AU which corresponds to 0.409 U/mL and the LLOQ was set to 0.029 AU , corresponding to 0.020 U/mL accordingly. The values for AU (absorbance units) are all given after mean blank subtraction of 0.058 AU .

Table 3. 6-point and 7-point linearity with/without PEG including deviations and r^2 ($n = 3$).

Linearity samples	Theoretical concentration [µg/mL]	Absorbance [AU] w/o blank subtraction mean \pm SD	Absorbance [AU] with blank subtraction	Calculated LDH concentration [µg/mL]	Calculated LDH concentration RSD [%]
KPL1 no PEG	0.149	1.033 \pm 0.04	0.974	0.147	-1.08
KPL2 no PEG	0.074	0.560 \pm 0.01	0.502	0.078	4.14
KLP3 no PEG	0.041	0.324 \pm 0.01	0.265	0.043	4.16
KLP4 no PEG	0.018	0.140 \pm 0.005	0.082	0.016	-14.92
KLP5 no PEG	0.012	0.110 \pm 0.002	0.051	0.011	-7.48
KLP6 no PEG	0.007	0.087 \pm 0.001	0.029	0.008	8.19
r^2	0.9983				
KPL1 PEG high	0.148	1.619 \pm 0.03	1.568	0.148	-0.24
KPL2 PEG high	0.114	1.391 \pm 0.04	1.340	0.110	-3.40
KLP3 PEG high	0.074	1.231 \pm 0.03	1.180	0.084	13.04
KLP4 PEG high	0.041	0.938 \pm 0.04	0.887	0.035	-13.32
r^2	0.9963				
KLP4 PEG low	0.041	0.938 \pm 0.04	0.887	0.041	-0.57
KLP5 PEG low	0.018	0.480 \pm 0.01	0.429	0.019	2.42
KLP6 PEG low	0.012	0.348 \pm 0.02	0.297	0.013	5.00
KLP7 PEG low	0.007	0.218 \pm 0.01	0.168	0.006	-11.29
r^2	0.9981				

PEG = polyethylene glycol, KLP = common name for calibration standards, AU = absorbance units, RSD = residual standard deviation, r^2 = correlation coefficient.

From the linearity with PEG (Table 3) all seven points fulfilled the specifications in a split linearity (the linear range is divided in a higher and lower division, which must intersect at one point). The linear range expanded again from 0.020 U/mL to 0.409 U/mL, with 0.113 U/mL (KLP4, corresponding to 0.938 AU) as

common point. The determination coefficient of both high/low was with 0.9963 and 0.9981 higher than the acceptance value of ≥ 0.99 . The deviations (RSD) ranging from 13.04% to -13.32% were within the acceptance limits of $\leq 15\%$. 1,568 AU was set as ULOQ, the LLOQ reached 0.168 AU. The system suitability test (SST) was carried out in six-fold determination (also $n = 3$) with a concentration of $0.077 \mu\text{g/mL}$ and resulted in a mean absorbance of 1.315 ± 0.08 AU. The deviation was with 5.94% within the acceptance limit of $\leq 15\%$. The corresponding calibration curves and plot of residues are shown in Fig. 4.

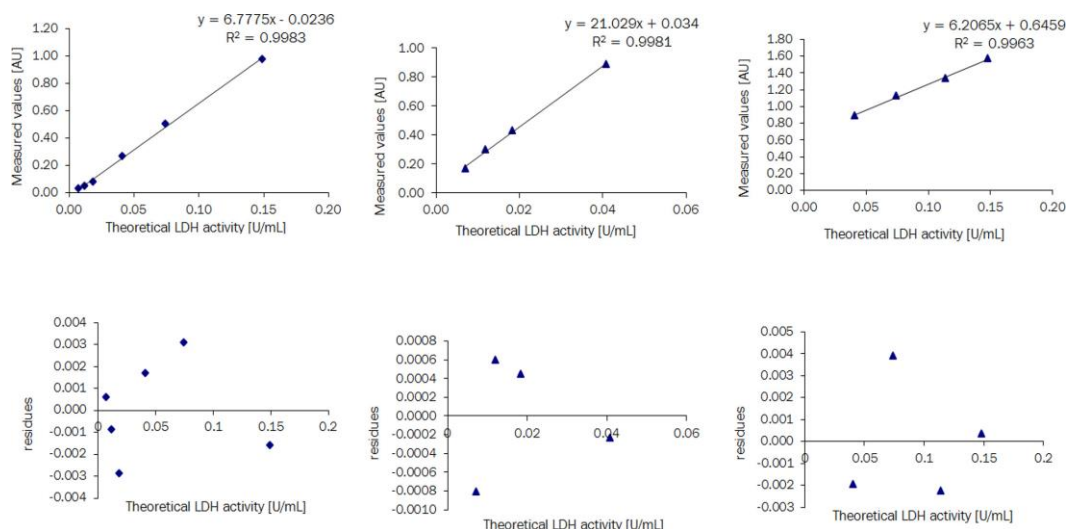


Fig. 4. Calibration curves and plot of residues. Left: 6 point calibration without PEG; middle: 7 point calibration with PEG, low part of the split linearity; right: 7 point calibration with PEG, high part of the split linearity ($n = 3$).

4. Discussion

In this study an LDH stability trial under different conditions was conducted and the LDH release test system was validated under bioanalytical aspects. In addition, a literatur review about nomenclature and specific properties of LDH isotypes was carried out.

After the clarification of LDH nomenclature in the literature, the research about specific features of LDH isotypes was considerably facilitated. Based on the studies of Lewis and Uitto [29, 30], LDH-5 is the main isoform in skin, followed by LDH-4. From the supplier of the recombinant LDH enzyme used in this study, the confirmation was obtained that also in this product, LDH-5 was the predominant isoform. This information is on one hand based on literature as the enzyme is derived from rabbit muscle, and LDH-5 is generally predominant in muscular tissue [28, 29]. On the other hand, an internal assay has also been conducted by the supplier but no more information was given due to

confidentiality reasons. As the claim of LDH-5 being predominant in muscle tissue could be supported during the first part of this study, the obtained information was considered as reliable.

Conflicting recommendations exist regarding stability of total LDH and especially isotypes LDH-4 and LDH-5. According to Collins et al. and de la Peña et al. [20, 22], LDH displays cryo-sensitivity and loses part of its activity after freezing, especially if subjected to repeated freeze-thaw cycles [20]. De la Peña identified 4 °C as preferable storage temperature for salivary LDH [22]. The predominant isoforms in saliva are LDH-4 and LDH-5, identical to cutaneous LDH isoforms [22, 30, 31]. In another study with salivary LDH [21], LDH was least stable at 4 °C, with room temperature obtaining a better recovery and the best results with storage at -20 °C. Services described activity loss of LDH-4 and LDH-5 with storage at 4 °C and -20 °C but reported longer preservation in frozen samples compared to refrigerated ones or those stored at room temperature [33]. Shain et al. did not find any instability/loss of activity of LDH-4 and LDH-5 after six weeks of storage, neither at 4 °C nor frozen at -20 °C [23]. Rohaya et al. investigated LDH stability at room temperature, 4 °C and -20 °C in presence of three different protectors [25]. The addition of PEG prevented degradation for two weeks at all three temperatures with a recovery of $\geq 98\%$, while glycerol seemed to be suitable only for frozen samples and ethylenediaminetetraacetic acid (EDTA) only for room temperature. Due to those contrasting results, an own stability study was conducted. As from Rohaya's study, PEG provided better results than glycerol and EDTA [25], PEG was chosen as protective agent as described. In this study, a low recovery for samples without PEG at 4 °C and -20 °C was obtained, with only slight variation between those two conditions, but differences between the concentrations. Storage at 4 °C in presence of PEG delayed the stability decrease for up to 2 days, being most visible in the high concentrated sample and partially supporting the finding of Rohaya et al. [25]. This PEG-derived preservation decreased, and from day 6 the recovery dropped down to the levels of storage without PEG. Storage of LDH at -20 °C in the presence of PEG displayed the highest recovery for the complete four week period. The results indicate, that the individual effects of concentration, temperature and cryoprotector are negligible while the combination of high concentration, low temperature and cryoprotection had a significant and

synergistic effect on the results. The best stability of LDH is therefore given at $-20\text{ }^{\circ}\text{C}$ in presence of PEG with a higher concentration while for the other conditions a marked instability was observed. These findings are in accordance with some researchers [21] but contrasting to others [20, 22, 23]. Some of these inconsistencies may stem from variation in sample handling, preparation and measurement due to different study protocols or enzyme composition, as already discussed by De la Peña [22]. Accordingly, as various recombinant LDH enzymes are available which differ in activity and isotype composition, their properties regarding stability and preferable storage temperature may differ as well, partially explaining the controversial results. Variation in laboratory procedures such as thawing of the enzyme before usage (thawing time, using heat or passive thawing at RT), incubation time (strongly depends on enzyme activity, usual variation from 5 min to 30 min), measurement wavelength, amount of cryoprotector, and equipment related restrictions may be another contributing factor.

The modified LDH assay used in this study was subjected to a partial validation to ensure sufficient accuracy and reproducibility. To be considered as valid, the correlation coefficient r^2 and deviations had to fulfill the corresponding acceptance criteria of the FDA bioanalytic guideline. Hereby, the focus was set on establishing a linear range, evaluating if a good correlation could be achieved and if the measurement system is suitable for the intended application. If these requirements were met, the investigation of further parameters was deemed unnecessary for this study. As with six, respectively seven points a full calibration curve was achieved, all the deviations for the linearity and SST were below 15 % and the r^2 was above 0.99 as specified in the guideline, it was concluded that the test system is validated for the application on *in vitro* viable skin models with which new insights about dermatological disease processes can be gained. This may improve therapeutic possibilities and the wellbeing of humans and animals.

Compared to several other methods of viability determination, the LDH release assay is non-destructive. A skin cultivation study over two weeks with regular measurements of the same skin specimen would not be possible with e.g. the MTT or WST test, for which the cell layers/tissue generally have to be damaged. The LDH release assay is also easy to use, non-expensive and fast. It is traditionally designed for cell culture systems and often used without being

validated first. With the adaption of the test system to tissue usage and partial validation, a higher degree of comparability and standardization is reached, providing an advantage over other methods.

Declarations

Author contribution statement

I.Bauhammer: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Sacha: Conceived and designed the experiments.

E. Haltner: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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Establishment of an *in vitro* viable skin model with human, porcine and canine skin and comparison of different media supplements

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Abstract

Transdermal drug delivery provides several advantages over conventional drug administration, such as the avoidance of first-pass metabolism and better patient compliance. *In vitro* research can abbreviate and facilitate the pharmaceutical development considerably compared to *in vivo* research as drug screening and clinical studies can be reduced. These advantages have led to the development of corresponding skin models. Viable *in vitro* cultured skin models are more useful than non-viable ones, due to the influence of skin metabolism on the results. While most *in vitro* studies concentrate on evaluating human-based models, the current study is designed for the investigation of both human and animal diseases. So far, there is little information available in the literature about viable animal skin cultures which are in fact intended for application in the veterinary and not the human field. Hence, the current study aims at filling this gap. For this *in vitro* viable cultured skin model, specimens of human, porcine and canine skin were cultured over two weeks under serum-free conditions. To evaluate the influence of medium supplementation on skin viability, two different supplement mixtures were compared with basic medium. The skin specimens were maintained at a viability-level >50% until the end of the study. From the tested supplements, the addition of bovine pituitary extract and epidermal growth factor increased skin viability whereas hydrocortisone and insulin induced a decrease. This *in vitro* viable cultured skin model may be a useful tool for the investigation of skin diseases, especially for the veterinary field.

1. Introduction

Transdermal drug delivery (TDD), compared to oral and parenteral drug administration, offers several advantages such as a decreased risk for toxicity and adverse effects, avoidance of first-pass metabolism and better patient compliance [2, 94]. In order to facilitate and abbreviate the pharmaceutical development process, an increasing trend to the transfer from *in vivo* to *in vitro* research has been observed during the last few decades [105, 106]. Simultaneously, the social acceptance for animal testing has remarkably decreased [10, 11, 107], resulting in corresponding changes in legislation. Examples include the 3R-principle in 1959, the European Centre for the Validation of Alternative Methods (ECVAM) established in 1991, the “Declaration of Bologna” in 1999 and the enactment of Registration, Evaluation and Authorization of Chemicals (REACH) in 2007 [12]. Consequently, *in vitro* research is being promoted as future standard, requiring the establishment of suitable models. The evaluation of percutaneous absorption (skin permeation) is essential in the design and development of various drug formulations. Only if a drug is able to overcome the outermost layer of the skin, the stratum corneum (SC), which constitutes the major part of skin barrier, can the drug be considered as a promising candidate for TDD. Whereas the SC poses a stronger barrier for hydrophilic substances, the permeation of lipophilic substances is limited by viable epidermis and dermis [108, 109]. However, the assessment of permeation is just one aspect of TDD and mainly useful for substance screening and defining rank orders to categorize drugs in different permeability classes. For the investigation of complex issues such as skin inflammation and disease, a more comprehensive approach has to be chosen. With chronic inflammatory and infectious skin diseases being a continuous problem [5-7] and decreasing efficacy of antibiotic treatment due to bacterial resistances [8, 9, 110, 111], new therapeutic options are imperative. The popular view of the SC as the only factor influencing skin absorption is not tenable anymore due to the detection of considerable metabolic capabilities of living skin [104, 112, 113]. Hence, viable skin is preferable for studying complex skin conditions [14]. One factor among others, affecting transdermal drug delivery, is skin resistance. A higher level of skin resistance is associated with skin integrity and an intact barrier [114], influencing methods such as iontophoresis [115]. This method employs a non-invasive electric current at low voltages for the delivery of

ionisable drugs into the skin [116]

Further skin properties need to be considered for TDD. Skin thickness varies considerably between different body regions [15, 56, 60, 117]. Human skin, e.g., is especially thin at the eyelids or directly behind the ears while on palms and soles it is very thick [56, 60]. Intensity of blood flow (*in vivo* or perfused models) to different skin locations proved to be an important parameter as well as functional properties, i.e. resistance toward stress or strain and the amount of elastic fibers [60, 118-120]. General metabolic activity of the skin and amounts of metabolic enzymes are also variable but tend to increase along with blood supply [121]. Fluctuations of the density of hair follicles between body regions but even more between different species (e.g. human vs. sheep) have to be taken into account as well as variations in lipid content and composition [15, 64]. All the aforementioned skin properties are also subject to considerable variation between human individuals depending on sex, body weight, age and ethnicity with variations up to 66 % [15, 117]. For this study, viable skin was chosen due to better predictability of *in vivo* conditions [117] and potential metabolic activity although no exact determination of the metabolic capacities was intended. If the skin has to be maintained viable over a prolonged time, the usage of an appropriate viability marker is crucial.

However, most of them are designed for cell culture and not for tissue, such as the standard assays MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and WST (water soluble tetrazolium salts). Furthermore, they are destructive to the tissue and often very time-consuming. [122, 123] The finally selected LDH (lactate dehydrogenase) release assay, however, is shorter, easy to use and non-destructive. Therefore it can be used for repeated measurements with the same skin specimen which is crucial for this study [124]. As this assay is also in principle designed for cell culture, a modified version, adapted to tissue [125] and validated according FDA (Food and Drug Administration, USA) guidelines [126], was used in the current study.

In most, if not all studies involving *in vitro* skin models, the focus has been on human research, with animal skin models as mere approximations of the properties of human skin, e.g. such as the pig ear model [14-16]. Therefore, although human skin was employed as well, this study aims to benefit the veterinary field by adding valuable information about *in vitro* cultured skin

models with viable animal skin which can scarcely be found in the literature [127].

Hence, a long-term cultured *in vitro* viable skin model from human, porcine and canine skin was established under simplest and serum-free conditions. Here, the aim was not human research but to fill the empty space in the literature regarding *ex vivo* cultured animal skin models and bridge the gap between human centered and animal centered research and medicine. Due to availability restrictions, the planned number of species for this study (including feline, equine and bovine skin) was not achieved. Therefore, porcine skin was not only selected for comparison to human skin, but also to represent the group of livestock animals. Canine skin represented the group of companion animals but was also chosen because of a known predisposition of dogs for skin diseases [5]. Several criteria for selection of the particular skin donors applied (same sex and age group, normal body weight). Only full-thickness trunk skin was considered. The selected body regions were determined by the human skin donor since only abdominal skin was available. Abdominal skin, however, was avoided for the canine donor due to mammary complexes and scar tissue following mastectomy. Back skin was also avoided due to pigmentation and higher amount of hair follicles. Therefore, skin from the flank/lateral abdominal region was taken. Back skin was chosen for the porcine skin specimen based on the results of Khiao et al. [128] who compared histological and functional properties of porcine skin from different anatomical regions with human abdominal skin. According to these results, both back and flank skin are most similar to human abdominal skin. However, due to skin injuries in the flank region of the pig, back skin was deemed as better option in this case.

The absence of nutrients provided by serum had to be compensated by adding other supplements to achieve better viability results. Therefore, the influence of supplementation with insulin, hydrocortisone, human epidermal growth factor (hEGF) and bovine pituitary extract (BPE) was evaluated. These four supplements were selected as they are known to have beneficial effects on skin and skin cells [129-133] which is why they are included in media for keratinocyte growth and proliferation (e.g Keratinocyte growth medium 2 from Promocell). The selection of these supplements was hence based on findings in the literature where their effects were described separately and in combination [132, 134]. Based on the

literature findings and due to practical reasons, insulin and hydrocortisone were evaluated as one supplement mixture and EGF plus BPE as another.

4. Materials & Methods

4.1. Skin preparation and cultivation

Human abdominal human skin (53 y/o, female, Caucasian) was obtained after informed consent of the patient according to the Declaration of Helsinki from esthetic surgery (Cabinet Dr. Pierre Sibille, Luxembourg, Luxemburg), porcine back skin (ca. 6 months old, female, Deutsche Landrasse) from a slaughterhouse (Schwamm und Cie, Saarbrücken) and canine flank/lateral abdominal skin (11 y/o, female, middle-sized mongrel) from a local veterinary practitioner (Dr. Norbert Paulus, Saarbrücken) after the dog's euthanasia with informed owner consent. All skin specimens were freshly excised and stored/transported for less than 2 hours at 4°C and high humidity to maintain their viability as high as possible. The subcutaneous fat was carefully removed with a scalpel, the tissue surface was rinsed with water and then gently dried. From each of the three skin specimens, nine full skin punches of 13 mm diameter were taken and weighed. They were cleaned under sterile conditions (sterile bench laminar airflow, Heto-Holten GmbH, Wettengel, Germany) with a mixture of phosphate buffered saline (PBS) solution (Merck Millipore, Darmstadt, Germany) and 70% ethanol (Waldeck GmbH & Co KG, Münster, Germany) and placed dermal side down in 12-well plates (Costar 3513 12-well plates from Corning Life Sciences, Kaiserslautern, Germany). From the nine punches per species, three skin punches were placed together in one plate. Three plates per species were cultivated using three different media, so that in total 27 skin punches have been cultivated as shown in figure 1.

One plate from each species was filled up to 0.7 mL per well with Dulbecco's modified Eagle's medium solution (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany), containing 3.7 g/L sodium bicarbonate (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) and glucose (VWR International GmbH, Darmstadt, Germany). 75.5 mg/L gentamycin sulfate (Merck Millipore, Darmstadt, Germany) were added from a stock solution of 15 g/L gentamycin sulfate. This solution is further referred to as basic medium. Another plate was filled up with the same

DMEM solution, but with an added supplementation of insulin and hydrocortisone, each 2 mL/L of DMEM (KGM single quotes, Lonza, Walkersville, USA), further referred to as supplement mixture 1. The third plate was filled up with DMEM solution and supplementation of bovine pituitary extract (BPE) 8 mL/L and human epidermal growth factor (hEGF) (KGM single quotes, Lonza, Walkersville, USA), 2 mL/L of DMEM, further referred to as supplement mixture 2 (see figure 1).

Graphical illustration of the sample distribution for cultivation

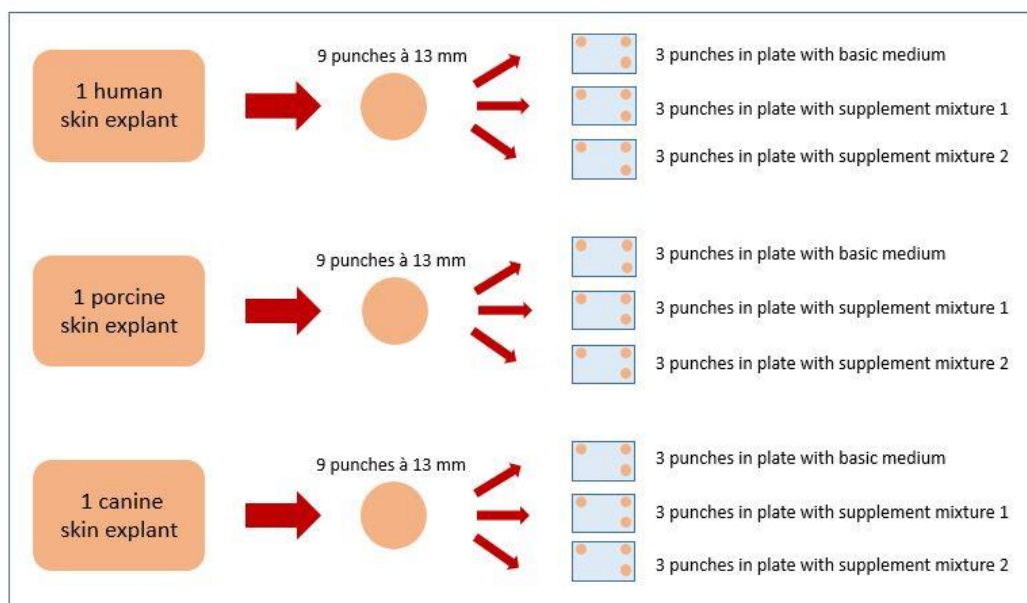


Figure 1: From the human skin specimen, 9 skin punches were made. These 9 punches were then distributed into three 12-well plates, three punches per plate. Each of the three plates was filled up with either basic medium, supplement mixture 1 or supplement mixture 2. The porcine and canine skin explants were treated accordingly.

The supplements were originally designed for keratinocyte growth medium. For the cultured skin model, a higher need of nutrients for full skin compared to cell monolayers was assumed, hence the recommended concentrations for cell culture were doubled, based on some ranges given in the literature [132, 134]. These concentrations may be optimized for receiving best results in future experiments but serve as working standard in this study. The exact concentration of the supplements themselves could not be determined as no further information was provided by the supplier (KGM single quotes: CC-4002E BPE, CC-4015E hEGF, CC-4021E insulin, CC-4031E hydrocortisone).

The SC (stratum corneum) of every skin punch remained uncovered with medium at the air-liquid interface. The skin explants were then cultivated over 14 days with daily sampling and medium change in a sterile incubator (Heracell Incubator, Heraeus, Hanau, Germany) at 37°C in 5 % CO₂/air to keep the pH of the medium at 7.4 [135] which is not only important for the cultured skin but also for the enzymatic reaction employed for measurement [136]. Photographs of the plates were taken every other day to monitor the medium levels and changes in the skin's appearance (see figure 2).

105 µL polyethylene glycol (PEG) 400 were added to the daily collected samples from medium supernatant containing LDH before storage at -20 °C for up to 4 weeks until measurement. These conditions were chosen according to the results of a previous stability study [126].

4.2. Controls

In order to quantify the highest possible LDH release from the skin of each species, a positive control was established for which a piece of skin was weighed, added to a defined amount of DMEM and mechanically destroyed with an immersion blender. After skin destruction, the suspension was filtered through a nylon net, the filtrate was centrifuged and the supernatant was aliquoted and stored at -20 °C for measurement. Measurement result of every positive control sample was considered as 100 % of the total LDH amount of this skin specimen. A negative control was obtained by heating one aliquot of positive control to 65 °C for 30 minutes [136] to denaturize all the contained LDH. The negative controls serve as blank sample for positive controls, i.e. the measured absorption of the negative control was subtracted from the absorption of positive controls to remove the influence of turbidity of the medium due to skin matrix. From the absorbance values of the positive control, the LDH activity per gram skin relative to those of the control was determined in order to calculate the viability of the skin in percent. 50% were set as limit, lower values were considered as indicators of a non-viable skin specimen.

4.3. Measurement by plate reader

Photometric measurement of the obtained medium samples for the quantification of LDH release into the medium was conducted with a plate reader (microplate reader Wallac 1420 Victor 2) using an absorbance wavelength of 450 nm (see figure 2). The generally cell-based LDH release assay was modified and validated for its usage on tissue beforehand [126].

For measurement, the samples were thawed at room temperature, shaken, diluted with DMEM into the linear range of the LDH calibration curve and pipetted with piston-stroke pipettes (Eppendorf GmbH, Wesseling, Germany) into a generic 96-well plate (here Nunclon Delta Surface, Thermo Fisher Scientific, Karlsruhe, Germany). Tubes from Eppendorf and Corning (Corning Life Sciences, Kaiserslautern, Germany) were used for sampling and measurement preparation. All the cultivation samples were measured in duplicate, except the control samples which were measured in triplicate. Reported values are mean values if not specified otherwise.

All calculations were performed using Microsoft Excel 2010.

4.4. Data analysis

Statistical evaluation was conducted using statistics software OriginPro 9.0 (Additive GmbH, Friedrichsdorf, Germany). Non-parametric analysis such as Kruskal-Wallis ANOVA (KWA), NPH k independent samples test (NPH) combining Kruskal-Wallis ANOVA and Mood's Median Test (MM) has been employed. A p -value of < 0.05 was considered significant. The exact p -value and degrees of freedom (DF) are reported.

Illustration of skin cultivation samples and LDH measurement

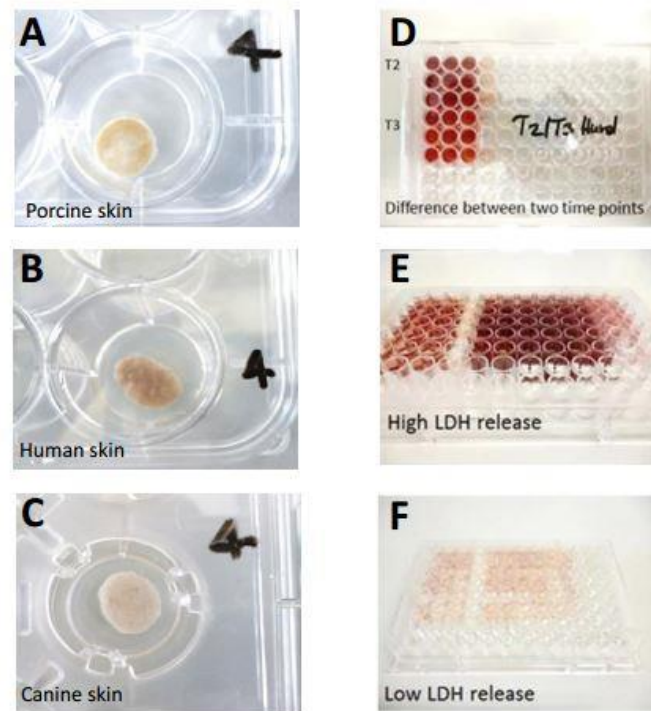


Figure 2: (A) porcine skin punch on cultivation day 7, well 4. (B) human skin punch on cultivation day 7, well 4. (C) canine skin punch on cultivation day 7, well 4.

(D) Exemplary LDH measurement to demonstrate the difference in LDH content between two timepoints (T 2 and 3) which is shown in the slight color change from T2 (upper nine wells) to T3 (lower nine wells). (E) Exemplary LDH measurement displaying high amounts of LDH (dark red). (F) Exemplary LDH measurement displaying low amounts of LDH (light red/pink).

The LDH measurements in panels D-F do not correlate with the skin punches in panels A-C.

5. Results

The LDH content of the positive control samples showed considerable variation between species. The human control reached 163.1 U/g skin, porcine control 124.8 U/g skin and canine control 57.3 U/g skin. The total LDH release over the whole cultivation time reflected the differences seen in control samples. With 76.19 ± 0.03 U/g skin, LDH release of human skin was the highest in comparison to the other species (Fig. 3) with significant differences between the three species (NPH: KWA $p=0.02732$ DF 2, MM $p=0.04285$ DF 2). Relative to the positive control, however, human skin was with 46.72% release in the middle, slightly lower than porcine skin. The total release of porcine skin was 60.66 ± 0.09 U/g skin while the relative release was the highest one with 48.60%. Canine skin

displayed a total release of 19.17 ± 0.3 U/g skin, considerably lower than the other ones. Also the relative release of canine skin was 33.47%, and thus lower than human and porcine samples with significant differences between the three species (NPH: KWA $p=0.00179$ DF 2, MM $p=0.02732$ DF 2).

Comparison of the total released LDH of each species at the end of the study with the relative LDH release as percentage of the positive control of each species

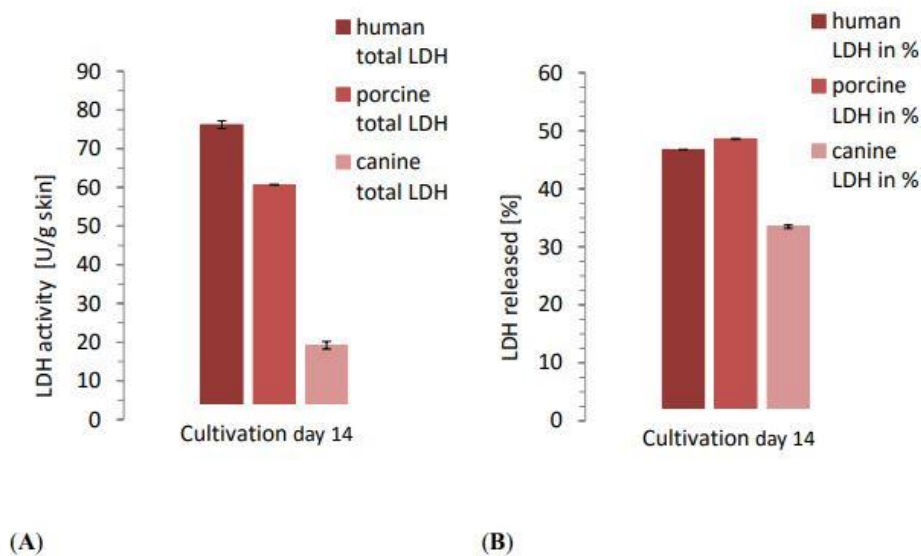


Figure 3: (A) Total amount of LDH released by all the human, porcine and canine skin samples in catalytic units per gram skin until end of cultivation (day 14). The columns are calculated from the cumulated sum (e.g. sum of LDH release of all human samples at timepoint d0+d1+d2+...d14; same for porcine and canine samples) of all the means from each species, respectively. (B) Amount of LDH released by all the human, porcine and canine skin samples as percentage of the corresponding positive control. Same principle for calculation applies as for panel A.

In the viability trend (Fig. 4 and Fig.5), the mean percentage of viability of the three investigated species already ranged from 83.26% (canine) over 73.76% (human) to 67.46% (porcine) on the first measurement time point, displaying an order that was maintained until day 7 (67,70% canine, 54.18% human, 52,13% porcine) and throughout the whole cultivation time. The curves of the different cultivation media for one species were similar in the case of porcine and canine skin (Fig.5 B and C) but not for human skin where the three curves exhibited a different behavior and intersected on the second day of cultivation (Fig.5 A). A rather rapid drop in viability occurred during the first two to three cultivation days, followed by a gradual decrease afterwards. This decrease happened until day

1 in porcine skin (mean), until day 2 for canine skin and until day 3 for human skin with a slope being slighter in the latter than the other ones (Fig. 5). The difference in viability between the three species at the beginning of the cultivation was not significant (NPH: KWA $p=0.36788$ DF 2, MM $p=0.22313$ DF 2) while the difference after seven cultivation days was significant (NPH: KWA $p=0.01832$ DF 2, MM $p=0.01111$ DF 2). The statistical species difference in viability for day 14 is reported with figure 6.

In the course of the first cultivation week (roughly until day 5 to 7) a marked decrease in viability was visible for each curve while afterwards either a plateau was reached (Fig. 4 B) or a slight further decrease could be observed (Fig. 4 A and C). The effect of the respective cultivation medium can be seen in figure 4, where for all three species, supplement mixture 1 obtained the lowest (Panel B) and supplement mixture 2 the highest viability results (Panel C) are shown.

Viability trend of the skin samples in percent over 14 days of cultivation,
comparing cultivation media

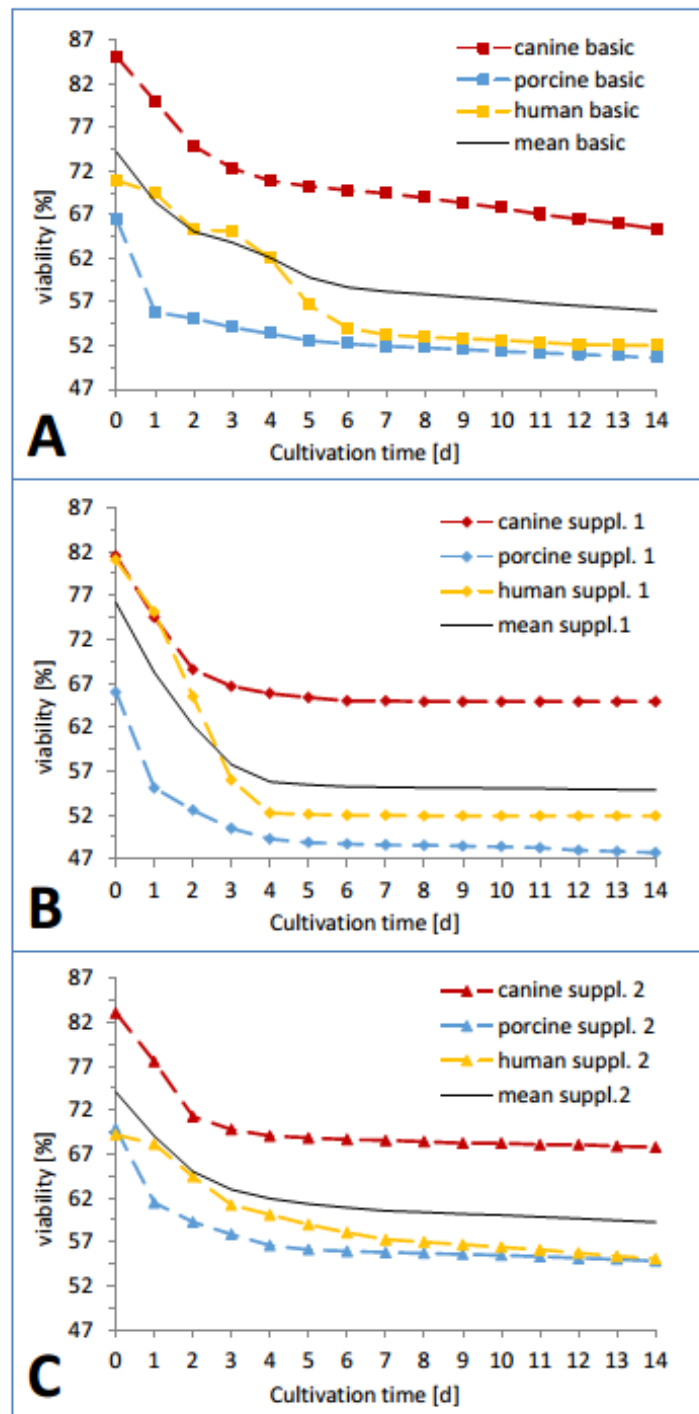


Figure 4: Viability trend (measurement timepoints connected via trend-lines) in percent relative to the corresponding positive control for the three investigated species: human (yellow), pig (blue) and dog (red) in order to compare differences between cultivation media. All the data points are mean values from three skin punches cultivated in the same condition, measured $n=2$. (A) basic medium (B) medium with added supplement mixture 1, (C) medium with added supplement mixture 2.

Viability trend of the skin samples in percent over 14 days of cultivation,
comparing species

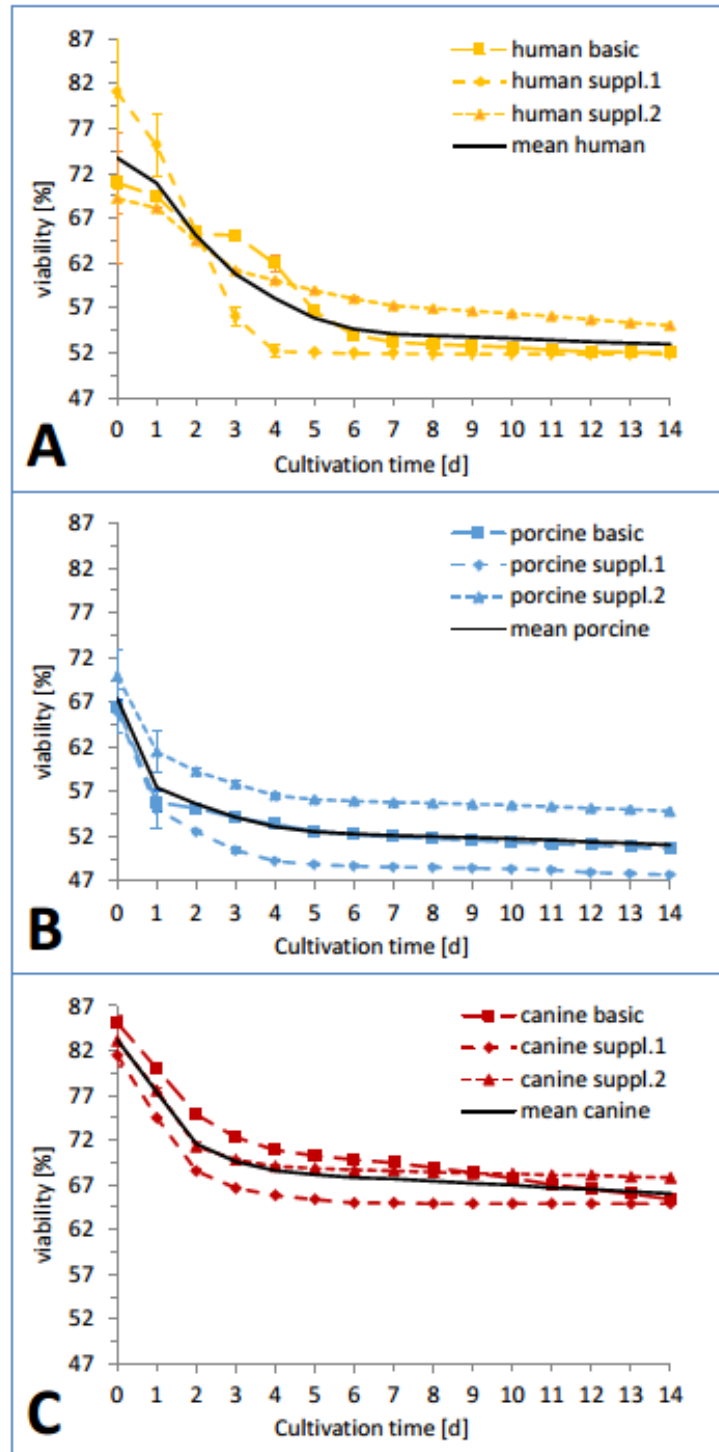


Figure 5: Viability trend (measurement timepoints connected via trend-lines) in percent relative to the corresponding positive control for the three investigated cultivation conditions: basic medium, supplement mixture 1 and supplement mixture 2 in order to compare differences between species. All the data points are mean values from three skin punches cultivated in the same condition, measured $n=2$.

(A) human skin, (B) porcine skin, (C) canine skin

Comparison of the viability of the skin samples at the end of the study

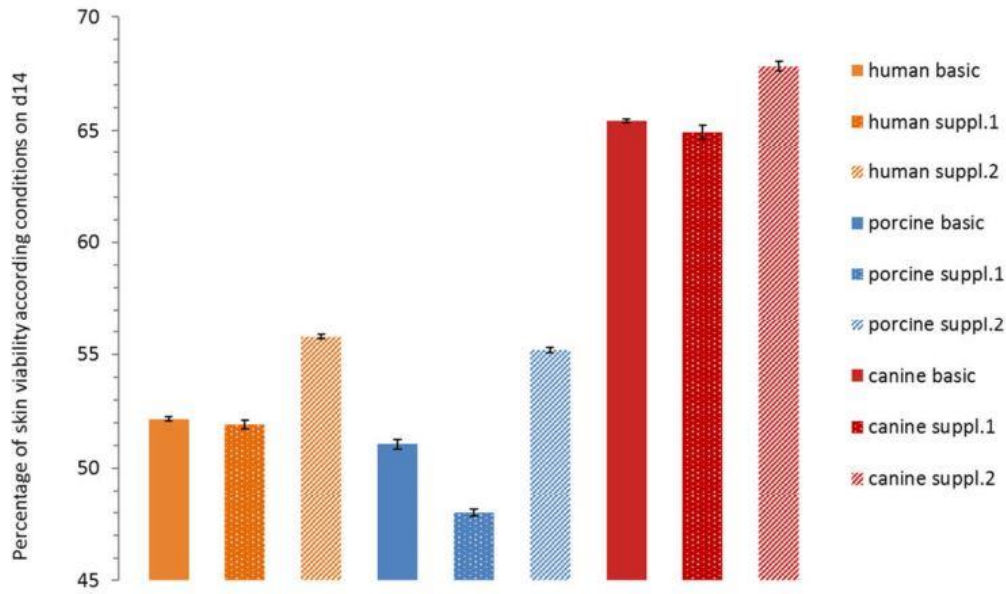


Figure 6: Remaining viability of the skin samples at the end of the study (day 14). Differences in viability levels between the three species: human (yellow), pig (blue) and dog (red) and between the three cultivation media: basic medium (plain), medium with supplement mixture 1 (dotted) and supplement mixture 2 (striped) are compared. The values are given as mean of each cultivation condition (e.g. human skin in basic medium).

The viability of each plate on the last day of cultivation is shown in fig. 6. Human skin in basic medium reached a viability of 52.16 ± 0.10 %. In supplemented medium it obtained a viability of 51.90 ± 0.20 % (suppl.1 = insulin+hydrocortisone) and 55.77 ± 0.10 % (suppl.2 = BPE+hEGF), respectively. Porcine skin viability ranged from 51.06 ± 0.21 % (basic medium) over 47.98 ± 0.15 % (suppl.1) to 55.17 ± 0.12 % (suppl.2). Canine skin viability extended from 65.43 ± 0.09 % (basic) over 64.93 ± 0.30 % (suppl.1) to 67.83 ± 0.22 %. The differences were significant between canine skin and human skin (KWA $p=0.04953$ DF1) and between canine and porcine skin (KWA $p=0.04953$ DF1), but not between human and porcine skin (KWA $p=0.05091$ DF2). The difference between all three conditions was again significant (MM $p=0.04285$ DF2). The absolute difference between the cultivation condition with the highest and the lowest viability were 2.9 % for canine, 3.78 % for human and 7.19 % for porcine skin. By referring to the corresponding highest value as 100 %, hence calculating the relative variance describing the ratio of the observed difference compared to the absolute values, canine skin showed 4.28 % difference, human skin 6.78 % and porcine skin 13.03 %. The variation within porcine skin was therefore about

double compared to human skin and four times higher than within canine skin.

The effects of medium supplements on viability were significant comparing suppl.1 with suppl.2 (MM $p=0.0455$ 1DF) and basic medium with suppl.2 for all three species (MM $p=0.0455$ 1DF). Comparison of basic medium with suppl.1 resulted in a significant difference only for porcine skin but not for the other two species (MM $p=0.0455$ DF1). However, these findings might have been more significant if a larger sample size was available.

6. Discussion

In this study, a viable *in vitro* model of cultured human, porcine and canine skin was established and cultivated for two weeks with regular viability measurements using a modified LDH release assay [125]. The total LDH content of each skin specimen and the influence of two different medium supplements on the skin integrity and viability were investigated.

By now, a large variety of skin models from human skin has been established, closely followed by different porcine models while for canine skin very scarce information can be found in the literature, emphasizing the importance of this research. One of the most common porcine cultured skin models is the pig ear skin model. The skin of a pig's ear is comparable to human skin in terms of skin thickness, lipids, hair follicle density and permeability [15]. Percutaneous absorption and skin metabolism of pig ear skin was evaluated by Jacques et al.[137] While the sample processing and cultivation conditions were similar to the current study, the skin was sectioned to a defined thickness with a dermatome (vs usage of full skin) and only a short-term culture of 48h was performed (vs. 14 days). Further examples include Scoglamiglio et al. [96] and Gomes et al.[97] who used pig ear skin for the evaluation of deformable liposomes and ethosomes or nanostructured lipid carriers, respectively. Porcine abdominal skin (dermatomed) was employed by Nagelreiter et al. [138]. One example of a dog skin model is described by Serra et al.[127] who developed a canine skin equivalent with isolated keratinocytes and fibroblasts which differentiated into a multilayer epidermis starting from day 15. A cultivation method with canine skin was reported by Abramo et al. [139] who used full-thickness adult canine skin in serum-free medium. These conditions are similar to the ones employed in the

current study, however, the skin was only maintained until day 7 and 4 mm skin biopsies have been collected in contrast to skin culture over 14 days and 13 mm punches in this study.

Skin specimens from the three species were cultured for two weeks. The skin could be maintained at minimum 50 % viability for the whole 14 days of cultivation which is a better result compared to other studies with a similar approach, such as described by Suarato et al, De Wever et al. and Castagnoli et al. [140-142]. De Wever investigated the NativeSkin® model where human skin biopsies from plastic surgery were placed in a solid nourishing matrix and were maintained viable for 7 days, using MTT assay and histologic characterization as viability markers. Castagnoli used human post-mortem allograft skin biopsies. Their viability was also assessed with the MTT assay. A viability level of the fresh skin of around 75 % was reported after 12-30h following harvesting which decreased to 40 % after 60h. After 6 days of cultivation, the viability further decreased to 0% which held also true for cryopreserved samples using 10 % DMSO. Storage at 4°C extended viability but did not prevent its decrease to 25 % after 15 days. Suarato, however, used explanted mouse skin in a 3D printed diffusion cell which could be maintained viable for maximum 24h, assessed again with the MTT assay.

Due to the different cultivation conditions (e.g. sample size, number of samples, cultivation medium) and the necessity for repeated measurements to assess the skin viability, the LDH assay was deemed a suitable alternative. The viability profile of the three cultivation conditions for each species showed a notable difference in viability starting from the first measurement which, however did not prove to be statistically significant. A sharp decrease in viability from the start until the first (pig), second (dog) and third (human) cultivation day is typical for this kind of tissue culture, also the following plateau or relatively flat interim period. A second decrease phase is reported in the literature [143, 144] but was not observed here.

The evaluation of the LDH content of human, porcine and canine skin based on the positive control samples revealed a rank order of LDH content as follows: human > porcine > canine skin. The total LDH release over the complete cultivation time reflected this. The relative LDH release of each skin specimen in relation to the positive control, however, was in the order porcine > human >

canine skin. This, combined with the rather low initial viability, might be a contributing factor to porcine skin yielding the lowest viability results at the end of cultivation. The low initial viability values could be due to the longer transport of the pig cadaver from the slaughterhouse to the place of processing, which was in another city. However, the differences in the initial viability levels were not significant and the viability of human skin after 14 days only slightly exceeded that of porcine skin, so that a major influence seems improbable. Over the whole study, the canine skin specimen maintained the highest viability.

Still, these results do not claim to prove a generally higher viability of canine skin which is not possible due to a notable amount of factors influencing skin properties as mentioned in the introduction. Although with human abdominal, canine flank skin and porcine back skin three physically adjacent regions (with assumed functional similarities) have been selected and the influence of sex, age and weight has been considered, variations in viability due to different anatomical and functional properties (e.g. skin thickness, lipid content and composition, absorption of nutrients etc.) could not be excluded. This may be especially the case considering canine skin, as morphological and functional analysis of porcine back skin showed great similarities to human abdominal skin [128]. However, there is little reliable information available about these properties in other animal skin and how and to which extent they may have affected the outcome of this study or about their influence on TDD in general. Therefore, a new concept such as employed in this study has to be based on a certain amount of assumptions and speculations which will have to be verified or falsified in the future.

It should also be taken into account that different skin models with rodent skin (e.g. rat, mouse, guinea pig and several hairless breeds of those) have been widely used as surrogates for human skin despite substantial differences in size, skin thickness, lipid composition, metabolism and skin barrier function [14, 15, 145]. In view of this, the current selection of species and body regions of the explants may be justified for the scope of such a pilot study.

The two supplement mixtures consisting of insulin plus hydrocortisone (suppl. 1) and BPE plus hEGF (suppl. 2) were selected according to Lasnitzky [146]. For all the samples, the medium supplement mixture 2 was the most effective in promoting health and viability of the skin specimens. This could be supported by a statistically significant difference between basic medium and supplement

mixture 1 compared to supplement mixture 2. This effect was significant in every species which makes usage and optimization of supplement mixture 2 the most promising approach for further studies. Among the cultivated samples, the porcine skin samples cultured with supplement mixture 1 failed as only ones to maintain a viability > 50 % until the end of the study and had therefore been deemed as non-viable. While for all the species, supplement mixture 1 had a certain negative effect on the viability even compared to basic medium, for porcine skin which generally expressed the highest relative variation between the three culture conditions, this effect was more pronounced and statistically significant. Therefore, supplement mixture 1 seems less suitable for further experiments. All the other skin samples (except porcine + supplement mixture 1) could be kept at a viability level of over 50 % until the end of the study.

In this study, no serum was used; hence some of the nutrients for the skin cells were introduced via these medium supplements. The positive effect of the supplement mixture 2 will have to be assessed in detail but may be due to the mitogen, proliferative and lifespan-extending effects of EGF and BPE on different cell types such as keratinocytes, which are described in the literature [130, 147-150]. The negative effect of the supplement mixture 1 is most likely induced by hydrocortisone, as this component is known to possibly having both positive and negative effects which are strongly dose-dependent [151]. The higher influence of supplement mixture 1 on porcine skin may therefore be explained by high levels of stress-induced corticoids in the body of the corresponding pig at the time of slaughter [152]. Together with the supplement, the resulting amount of hydrocortisone may have been high enough to induce a viability decrease. However, choosing a lower concentration of hydrocortisone might be an option to be considered.

7. Conclusions

This *in vitro* viable cultured skin model is a first step towards the investigation of canine and porcine skin diseases. In its current shape, it may be employed for topical application studies, percutaneous absorption and, due to its viability, basic cutaneous research, e.g. keratinocyte reactions to irritation. Upon further optimization, it may be used for assessment of skin metabolism which can be essential when applying xenobiotics (e.g. drugs) to the skin [137]. New cosmetic products or nanoformulations may be tested based on this model as well [153]. For these purposes, the skin viability and its structural integrity are crucial. Therefore, skin cultivated with the supplement mixture 2 is the most suitable candidate between the three tested conditions for further research. An increase of the current viability levels is planned for the future. Compared to the first step of this research [125], the life span of the skin has already been almost doubled. Different concentrations of the supplements, other cultivation media and supplements may be tested for further viability improvement.

The development of a complex disease model may later be based on the current *in vitro* cultivated skin model, being especially relevant for canine skin as only relatively few models exist. [127]. Introduction of skin from other species may considerably expand its scope. Skin properties and permeability may be evaluated and categorized with the aim to create a comparable data set and standards similar to those in human medicine.

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IV. DISCUSSION

A viable *in vitro* cultivated skin model was established, first with human skin, and then expanded to porcine and canine skin. In order to assess the viability of the skin, a commercial LDH release assay was adapted from employment in cell culture systems to tissue usage. This adapted LDH assay was then validated according to basic criteria of the FDA bioanalytics guideline (version of 2002 with respect to modifications in the draft version from 2013). Instability of the LDH enzyme used for the validation and the skin samples during the first measurements led to a stability study to determine the best storage conditions of LDH enzyme. In this stability study, usage of PEG as cryoprotector had a significant influence on the stability, consequently the validation was repeated with LDH in a medium containing PEG. The mixed species skin model was intended for the comparison of the viability and LDH release profile of the different species and to evaluate the influence of two media supplement mixtures on the skin viability.

It was important, to select viable skin for the skin model to be able to assess more than just the permeation and passive barrier properties of the skin, and to expand the application of the model for research which relies also on information about the metabolic capacities of living skin [103]. Culture conditions aimed to imitate natural conditions as close as possible to ensure “well being” of the skin. The LDH release profile of the human skin model was similar to comparable studies with human skin [143, 154]. A first peak could be linked to direct necrosis, mostly resulting from excision and handling [144] and a second peak marked the time point of maximum apoptosis rate. By maintaining the skin specimens viable for eight days, a middle range was achieved between shorter and longer cultivation times described by other authors [143, 154]. Due to the simpler cultivation conditions and experimental setup of the current study, these results are sufficiently reliable and acceptable as foundation for further studies. Simple conditions keep costs and complications as low as possible. This way, other laboratories can reproduce the work without the need for expensive compounds and devices or specifically trained personnel. Furthermore, the likelihood of human error is decreased. As bovine serum is a non-standardized natural compound with varying composition, its effects on skin behavior and viability

cannot be exactly determined [155, 156]. Thus, the cultivation was conducted without this specific compound. In most serum-free cultures, serum or some of its protective and proliferative effects are replaced by various medium supplements. This study focused on high standardization and separate evaluation of each compound, to see if cultivation of viable skin is feasible under those conditions at all and for how long. The validation of the adapted LDH release assay according to FDA criteria was another way to standardize the study and increase reproducibility, features which are lacking in many other studies [14, 15].

The same also holds true for the different and conflicting stability results for the LDH enzyme in the relevant literature. The respective LDH subtype or composition has to be considered, with different properties regarding stability, temperature and activity [136, 157, 158]. In addition, the medium in which the LDH is measured has to be considered as well [136]. Plasma provides other features than saliva or cultivation medium supernatant. The specific activity of the enzyme has to be determined at any time, due to large batch to batch variations [124, 136]. Correspondingly, the incubation time of the samples has to be adapted to the catalytic activity of the enzyme as the reaction continues as long as substrate is available [136]. This may lead to significantly different results even under the same conditions. Thawing of frozen enzyme should be done gently over time at room temperature or with any heating system. However, heating up the enzyme too much results in loss of activity due to denaturation. In contrast, with insufficient warming the preferred temperature range of the enzyme is not reached and the reaction is slowed down. Either leads to incorrect and unreliable results [136]. These examples are some of the reasons, why a general statement about any results is difficult when the exact experimental conditions are not apparent in the publications, especially regarding enzyme assays [136]. This is why in our studies each step was described as detailed as possible in order to provide the information required for optimal reproducibility and standardization [102].

For the expanded skin model, a variety of animal species were possible as skin source. Human skin was used for the investigation of human skin diseases but also to compare the results of this study with others to ensure accuracy. Porcine skin was also chosen for comparison reasons, because it is most similar to human skin [15], and as a representative for skin diseases in livestock. Canine skin was chosen, as dogs are common pets which are reported to show a general disposition

for skin diseases [5].

The results of the studies about both skin models leave some questions open. While the human skin model could be maintained viable over eight days, the mixed model could be kept viable over two weeks even without supplementation. This might be due to a change in the protocol. For the first human-only model, the cultivation medium was changed every day except weekends. So for two consecutive days the medium was not changed. In the second model a change of medium occurred every day. While for cell cultures this is rather usual, it could be that due to the higher density of cells more nutrients are used and fresh medium is required every day. In addition, LDH-containing samples were initially stored under non-ideal conditions and thus instable, as found out during the stability study [157]. However, instability of LDH would lead to decreased enzyme activity and hence higher calculated viability values (due to the inverse correlation of viability and amount of released LDH) [144], so this is unlikely. Therefore, these variations seem to be most likely due to the different medium change schedule and constitute the only part of the study which is not comparable regarding intra- and interstudy comparability.

In the mixed skin model, canine skin exhibited by far the highest viability over the whole cultivation time. There may be several possible reasons for this. Thickness of full skin, epidermis and SC is very variable between species, breeds, individuals and body regions [65]. Although some of the potential influencing factors such as sex, age and weight were taken into account, other factors such as skin thickness or lipid content and composition could not be completely excluded. Flank skin of dogs is relatively thin [159]. In addition, this was densely haired skin (although a region with rather low amount of hair follicles was selected) and thus the skin is thinner than that of more sparsely haired porcine (back) and human (abdominal) skin. Thinner skin means less cells and therefore a better transport of nutrients through the tissue which also uses less of them, so that the skin does not suffer between medium change time points. This would correspond to the possible explanation, why the first skin model worked badly with medium unchanged over the weekend. This assumption is further supported by the lowest viability of porcine skin which was the thickest of the three. Another skin-related characteristic influencing viability may be its lipid content and composition. Porcine skin was visibly the fattiest skin while canine skin seemed to be least

fatty. Reportedly, canine skin lacks epithelial lipid plugs, contributing to the relatively weak skin barrier of dogs [5]. Another factor to consider is the skin surface pH which is usually rather alkaline for canine skin with values from 5.5 to 8.8 [159] and more acidic in the case of human (4.5-6.0) and porcine skin (slightly more basic than human, around 6.0 to 7.0) [160]. Whether the higher pH of canine skin may provide protective properties could not be clarified.

One further point to consider is the time between surgery/slaughter/euthanasia and arrival of the skin in the laboratory. Human skin was cooled down immediately after surgery and the excised skin was transported in cool boxes for approximately two hours. This procedure was highly standardized. Canine skin was collected directly after euthanasia, cooled and wrapped in a damp cloth and transported in a cool box for approximately one hour. This was also highly standardized. Porcine skin, however, was obtained from a local slaughterhouse in another city. There is no information available as to when exactly this respective pig was slaughtered (early morning hours) nor when the skin was excised. The excised skin was subsequently transported to a local company (around 30 min), from where it was collected and transported in a cool box to the laboratory (another 30 min) to arrive at 8 am. This leaves a longer time span of around four hours, if it is assumed that the pig was slaughtered at 4 am. The procedure and conditions were not completely standardised. Therefore it is possible, that the viability of porcine skin is underestimated in this study. With the current results, the respective viability of each skin corresponds to its transportation time and conditions.

Regarding the negative effect of the cultivation medium supplement mixture 1, several causes are possible. For insulin, no information about adverse effects on skin cultivation could be found. For hydrocortisone, however, there is evidence for a negative correlation to skin viability. It delayed wound healing in one study with mice [151] for about one week, showing antiregenerative effects. This is supported by other studies, where hydrocortisone in the cultivation medium reduced cell proliferation of especially keratinocytes, decreased skin thickness and downregulated the metabolism of collagen tissue [161]. These effects were only observed if the concentration of hydrocortisone exceeded the physiological values toward a therapeutically active concentration. Although the supplement concentrations in this study are all based on recommendations from relevant literature, the bad performance of supplement mixture 1 may be due to high

hydrocortisone concentrations. This effect may be especially pronounced for porcine skin, as a high amount of stress-induced corticoids were circulating in the body already at the time of slaughter [152], remaining in the skin. Together with the supplement, a relatively high dose may have been reached and the originally beneficial effect may have turned into an adverse one. EGF and BPE were described to have only positive effects which could be further supported by obtaining the best viability results with this medium in this study [150].

With the establishment of this mixed species viable skin model, an essential step towards a higher degree of standardization and comparability was taken. This model can be employed for generic skin absorption studies both with viable and non-viable skin, but may find its main purpose in cutaneous basic research. The development of a disease model may later be based on this model. Furthermore, the insights and knowledge gained may be used in the development of cosmetic products as well.

The model in its current shape is already a handy and useful instrument for pharmaceutical drug development. It is also intended to be employed for the development and optimization of various nanoformulations for the treatment of inflammatory skin diseases, exceeding the scope of this thesis.

V. SUMMARY

Compared to other drug delivery systems, transdermal drug delivery has several advantages such as avoiding first-pass effect, lower risk of toxicity and higher patient compliance. While pharmaceutical drug development *in vivo* is complicated and expensive, *in vitro* it may be considerably facilitated, and, from an ethical point of view, it minimizes animal experimentation. Although for *in vitro* drug development various models exist, validated and standardized model systems are lacking, particularly in veterinary drug development. Due to the high prevalence of skin diseases in humans and animals, this dissertation focuses on the development and validation of an *in vitro* viable human and animal skin model as basis for dermatological drug development. During the first experimental phase, a human viable skin model was developed with the LDH release assay as selected viability marker. This assay was adapted to tissue usage as it is originally designed for cell culture. The modified assay was then validated according to FDA bioanalytical guidelines to assess its accuracy and reproducibility. After detection of an instability of the LDH enzyme during the first few measurements, a stability study was carried out to assess suitable storage conditions for the enzyme and skin samples. In the second experimental part, the scope of the human model was extended to a mixed model with human, porcine and canine skin, simultaneously testing the effect of two medium supplements on the skin viability. The human skin samples from the first model were maintained viable for 8 days, cultivated in basic DMEM in AIC at 37°C/5% CO₂. The modified LDH assay was successfully validated. The LDH samples were stable stored at -20°C with cryoprotector (PEG). The skin specimens of the mixed model were maintained viable for 14 days in basic/supplemented DMEM. Canine skin had the highest viability, followed by human and then porcine skin. Supplement mixture 2 had a positive effect on all the samples while mixture 1 showed a negative effect. The influence on porcine skin was the strongest. The successfully established skin model provides a basis for further refinement, but is already a useful tool for human and veterinary drug development.

VI. ZUSAMMENFASSUNG

Das transdermale therapeutische System hat gegenüber anderen Systemen einige Vorteile wie zB. Vermeidung einer vorzeitigen Metabolisierung in der Leber, geringeres Toxizitätsrisiko und bessere Annahme durch Patienten. Während die Medikamentenentwicklung *in vivo* kompliziert und teuer ist, wird diese durch einen Wechsel zur *in vitro* Entwicklung deutlich vereinfacht. Dies ist auch unter ethischen Gesichtspunkten von Vorteil, da so auf Tierversuche verzichtet wird. Bis dato existieren zwar verschiedenste Modellsysteme, sie sind aber meistens nicht ausreichend validiert und standardisiert. Dies gilt insbesondere für die Medikamentenentwicklung für Tiere. Aufgrund der Häufigkeit von Hautkrankheiten bei Mensch und Tier konzentriert sich diese Dissertation auf die Entwicklung und Validierung eines viablen Hautmodells mit menschlicher und tierischer Haut, um eine Basis für die dermatologische Medikamentenentwicklung zu schaffen. Im ersten experimentellen Teil wurde ein humanes Hautmodell entwickelt, wofür der LDH-Test zur Viabilitätsbestimmung ausgewählt wurde. Dieser Test, ursprünglich für die Zellkultur gedacht, wurde für die Anwendung an komplexen Geweben optimiert und danach nach den Vorgaben der FDA-Guidelines validiert. Wegen Instabilität des LDH-Enzyms wurde eine Stabilitätsstudie durchgeführt, um die besten Lagerungsbedingungen für das Enzym und die Hautproben zu bestimmen. Im zweiten Experimententeil wurde das humane Hautmodell um Schweine- und Hundehaut erweitert, bei gleichzeitiger Untersuchung des Einflusses zweier Mediumsupplemente auf die Viabilität. Die Hautproben des ersten Modells konnten für 8 Tage am Leben erhalten werden, kultiviert in DMEM als AIC bei 37°C/5% CO₂. Die LDH-Proben erwiesen sich als stabil bei -20°C mit PEG als Gefrierschutz. Die Hautproben des gemischten Modells konnten 14 Tage lebendig gehalten werden, kultiviert in original und supplementiertem DMEM. Hundehaut erzielte die höchste Viabilität, gefolgt von Mensch und Schwein. Die Supplementmischung 2 zeigte einen positiven Effekt auf die Hautviabilität, wohingegen Mischung 1 einen negativen Einfluss hatte, welcher beim Schwein am ausgeprägtesten war. Das erfolgreich entwickelte Hautmodell kann weiter verbessert werden, ist jedoch bereits ein nützliches Instrument für die human- und veterinärmedizinische Pharmazeutik.

VII. LITERATURVERZEICHNIS

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VIII. APPENDIX

1. Skin diseases

Skin diseases belong to a group of the most complex disorders. Their etiology can be multifactorial and often remains partially or completely unclear [162-164]. Genetic components, microorganisms, environmental and behavioral factors interact with each other in a way that still leaves questions open [162, 163]. In these cases, therapy focuses on relieving symptoms rather than being able to cure the underlying cause [164, 165]. As both human and animal dermatologic diseases are currently remaining on a high level or increase [5-7, 166], much more research and new therapeutic options are currently required and will be in future.

1.1. Human skin diseases

With skin being the largest organ of our body it cannot be avoided that also this organ, like many others, becomes diseased from time to time. As border between us and our environment the skin is continuously exposed to various and potentially detrimental influences, both from the outside world but also from inside the body. Toxins, chemicals and other irritants do not only affect our body by direct skin contact but also by incorporation, e.g. via nutrition. Among other factors they can influence the sensitive hormonal equilibrium and contribute to emerging allergies and intolerances. In recent years, a worldwide increasing trend in skin disorders of such origins was observed [6, 7, 166]. Another alarming trend is the epidemic of antibiotic resistances of pathogens like *Staphylococcus aureus* and many others [111, 167, 168]. One example of such a disease, combining hormonal, inflammatory and bacterial causative aspects, is acne vulgaris:

1.1.1. Acne vulgaris

Prevalence and description:

Acne vulgaris belongs to the most commonly seen inflammatory skin diseases. Affecting around 80% of adolescents, the disease often persists throughout many years of adulthood. The acne lesions are located in the pilosebaceous units of the face and back and can result in various degrees of hyperpigmentation and scarring [162, 163]. Even more than to the physiological symptoms and consequences of this disease, attention should be paid to the psychological effects, as this skin

condition can cause immense impact on psychological well-being, leading to anxiety, social isolation, clinical depression and eventually even suicidal tendency, while the severity of the disease does not necessarily correlate with the extent of psychological suffering [163].

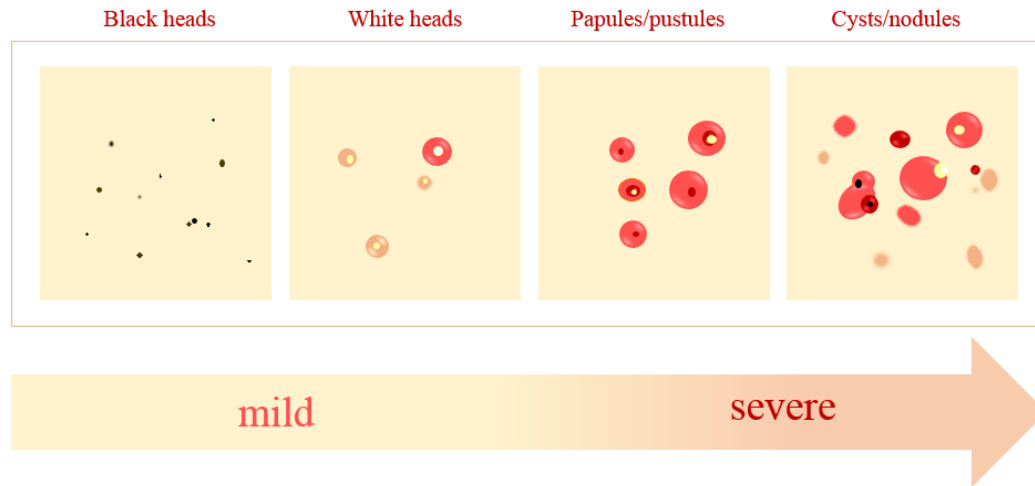


Fig. 19: The four stages of acne: mild, moderate, moderate-severe and severe.

Etiology and pathogenesis:

The pathology of acne vulgaris is very complex and multifactorial and therefore not yet completely clarified but four main factors in the pathogenesis of acne can be defined:

- Increased sebum production, influenced by androgens
- Changes in keratinization within the pilosebaceous unit which eventually lead to comedones through building up sebum inside the blocked duct.
- (Hyper)proliferation of the normal skin inhabitant *Propionibacterium acnes*
- Perifollicular inflammation

Genetic and further hormonal factors are supposed to contribute to acne pathogenesis as well [163]. The physical appearance of the disease condition can be differentiated in mild, moderate and severe acne, a classification which serves monitoring disease development and choice of therapy. The stages of acne lesions are described as micro-comedos without visible inflammation, followed by closed comedones which open up and become clearly inflamed papules, pustules, nodules and cysts which might leave scars and pigmentary changes behind [110]. Contrary to the conventional perception of acne pathogenesis according to which inflammation results from bacterial colonization of the pilosebaceous duct,

evidence arose, that, in fact, inflammation might be the precursor of all further happening. While the underlying immunochemical processes for the onset and aggravation of the inflammation are still subject to research, it has been shown, that *P. acnes* is not always involved in the development of inflammation and other pathways are existent [162].

The sebaceous gland might be the driving force in comedogenesis and inflammation. Elevated levels of pro-inflammatory cytokines such as IL-1 α , IL-1 β , EGF and TGF α are expressed by the sebaceous glands which lead to hypercornification and disorganization of the keratinocytes of the sebaceous unit. Genes for expression of proinflammatory mediators have been shown to be upregulated in skin with acne lesions, compared to normal skin. Increased sebum production contributes to the inflammation as sebum lipids display proinflammatory properties. The involvement of *P. acnes* might not be the source of inflammation but does propagate and aggravate it by triggering an innate immune response via activation of monocyte TLR-2 (Toll-like receptor 2), resulting in expression of further proinflammatory cytokines IL-8 and IL-12.

A neuronal influence on the development of acne lesions has to be considered as well. An elevated expression of CRH (corticotropin releasing hormone), MC-1R (Melanocortin-1 receptor) and SP (substance P) could be detected in skin with acne lesions. All three neuropeptides are involved in either stress response and or neurogenic inflammation and might be essential for stress-induced acne [162].

Therapy:

All these factors contributing to the etiology of acne should be also considered in the selection of an appropriate therapy. The conventional treatment with antibiotics has next to considerable side effects also the difficulty of emerging resistances. Focus on direct anti-inflammatory treatment with topical retinoids (e.g. Tretinoin) and benzoyl peroxide, in combination with antibiotics or without, has provided better results, but also here adverse effects especially in long term treatment can occur and still some patients are not responding to the medication [110, 111, 163].

1.2. Animal skin diseases

Animal skin diseases are often caused by parasitic, fungal or microbial infections [169-171]. Besides zoonotic risk, animal skin diseases are also a financial factor for producers and owners. Weight loss, delay in growth, low fertility, low milk

production, aggressive or auto-aggressive behavior and high mortality are some effects of acute and chronic skin diseases in livestock [172-174]. For companion animal-owners, long-term treatment, special foods and hospitalization are challenging as well [175].

1.2.1. Companion animals (e.g. cat and dog)

Small animals like cat and dog advanced over the years from livestock as herding and guard dog or professional mice-killer to family members. As such, they share human environment and lifestyle more closely than other animals. Unsurprisingly, allergies and adverse reaction disorders toward different stimuli have increased in prevalence and importance for both of them [5-7, 166, 176]. So does Atopic dermatitis affect human, dog and cat, as well as food allergies do. While some parasitic and bacterial diseases of small animals do not concern human, others do and with cutaneous leishmaniosis another example of a “shared” disease can be stated.

1.2.1.1. Why dogs are especially susceptible for skin diseases

Dogs show an over-average predisposition for skin disease, especially concerning bacterial skin infections. This is due to some properties of their skin even when the dog is completely healthy, but the risk is incomparably increased for a dog with an underlying disease such as atopic dermatitis, demodicosis etc. Normal dog skin has a very fragile skin barrier, a rather alkaline pH and lacks a follicular lipid plug, which can act as a drain stopper. In atopic dermatitis dog skin, the skin barrier is further weakened to almost non-existent and a component of the innate immune system, the so-called defensins, might be reduced.

The resulting pyoderma is induced by *Staphylococcus pseudintermedius* as main pathogen but *Staphylococcus schleiferi*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* can be involved as well. Since the commonly known *S. aureus* and recently also *S. pseudintermedius* have gained methicillin resistance, treatment became even more challenging.

Pyoderma can be categorized as following and can include various types of lesions and levels of pain and itching.

- surface pyoderma
- superficial pyoderma including
 - Impetigo

- Bacterial folliculitis
- Bacterial overgrowth syndrome
- Deep pyoderma including
 - Localized furunculosis
 - Generalized furunculosis/cellulitis

The different forms are treated with topical or systemic antibiotics depending on severity and bathing/spraying with chlorhexidine solutions. A sensitivity test for the antibiotic to be used is recommended [5].

1.2.1.2. Atopic dermatitis

Dog: Canine atopic dermatitis (CAD) [177] is a very common allergic skin disease of the dog, characterized by inflammation, potentially intense pruritus (itching) and associated with IgE antibodies to environmental allergens. The lack of a certain biomarker or metabolic profile and of pathognomonic clinical presentation makes diagnosis rather difficult. The involvement of genetic factors and the diversity of appearance in lesions and stage as well as possible secondary infections or further flare factors enhance this problem even more [164].

CAD usually starts with pruritus which elicits rubbing, scratching, licking and excessive grooming in the animal. The itch can be seasonal or non-seasonal depending on the allergen. Most commonly affected areas are the face, ear pinnae, axillae, perineal and inguinal region and the distal (lower) extremities. In beginning stage the pruritus can be exhibited without any lesions or primary lesions such as erythema and papules. In the further course of the disease, secondary lesions, e.g. alopecia, crusting, seborrhea, excoriations etc. are inflicted to the dog by self-trauma, chronic inflammation and secondary infections.

Before the diagnosis of atopic dermatitis, several differential diagnoses have to be excluded, as there are to consider:

- Ectoparasitoses (flea or mite infestations such as Demodicosis, Cheyletiellosis, Pediculosis, nasal mites, *Sarcoptes scabiei* etc.)
- Microbial infections (pyoderma due to malassezia, staphylococcus spp.)
- Other allergies (flea/insect bite allergy, food intolerance/allergy)

As of this wide range of similar diseases – which might be all concurrent diseases next to CAD as well – the so-called “work up” of the patient is crucial. By flea combing, hair plucking, skin scraping and cytology of skin and ear samples or in

some cases therapy upon suspicion to confirm or negate a previous assumption, the differentials may be ruled out one by one over time.



Fig. 20: Atopic dog before (left) and after (right) 2 weeks treatment:

Decreased redness in the right picture, dog can be handled without constraint=decreased itching/pain (Pictures friendly provided by Birgit Heichele)

CAD is not curable and treatment remains symptomatic, especially as long as the disease-causing allergen is not found. Possible allergens include pollen, dust mites, food components and others. The reactivity of the skin toward different allergens is evaluated by intradermal testing (IDT) or detection of IgE by allergen-specific IgE serology (ASIS) [164] After detection of the responsible allergen(s), allergen-specific immunotherapy can be an effective treatment option [165].

Cat: Although there is a lot of similarity on first sight in allergic cats and dogs, it should be always considered, that a cat is not a small dog and disease origin, clinical signs, treatment and complications can be strikingly different.

While the other two hypersensitivity diseases, insect bite allergy/hypersensitivity and cutaneous adverse food reaction (CAFR), are similar between both species, atopic dermatitis is different. Not much is known about the pathogenesis in the cat

but the lack of conclusive evidence of the influence of IgE on it led to controversy about whether the disease can be called atopic dermatitis or not. Therefore, the more exact term “non-flea non-food hypersensitivity dermatitis” (NFNFD) was introduced. Although etiology and pathogenesis of this disease are still not completely clear, a genetic involvement is likely. Also the pattern of inflammatory cells is comparable to dog and human. The exclusion of parasitary infections and other allergies has to be done in accordance. The concurrent pyoderma and *Malassezia species* overgrowth commonly found in dogs was reported less frequently in cats with NFNFD/atopic syndrome. If this is due to a relative resistance of cats compared to dogs, supposedly because of decreased bacterial adherence to the corneocytes, or if it is a result of overlooking the less prominent lesions in the cat, is so far not distinguishable. The role of skin barrier function is also for cats as well as people and dogs, recognized as a major factor in disease development.

Common symptoms in cats are pruritus and inflammation, involving head, neck and pinnae, causing excoriations, self-induced alopecia, miliary dermatitis, eosinophilic lesions and non-painful ulcerations. Next to the cutaneous reaction, however, other symptoms can occur, including allergic otitis, sinusitis and conjunctivitis, sneezing and feline asthma.

Treatment is symptomatic as for dog and human, including fatty acid supplementation, antihistamines or glucocorticoids. Allergen-specific immunotherapy is also possible. IDT and ASIS can be used as a guide for immunotherapy, but do not serve as a definite diagnostic tool [165].

1.2.1.3. Flea bite hypersensitivity/Flea allergic dermatitis (FAD)

FAD is a very common dermatologic disease of cats and dogs. In moderate and cold climate present just during summer, the disease can persist throughout the year in warm and humid climate [178]. Fleas inject their saliva while feeding into the host animal. This saliva contains various histamine-like components, enzymes, amino acids and polypeptides which can induce hypersensitivity. Dogs which are not used to flea bites develop immediate (15-20 min) or delayed (24-48h) reactions, sometimes both, with elevated levels of circulating IgE and IgG antibodies [178, 179]. If the dog is continuously exposed to fleas, they have low level of these antibodies and do not display skin reactions or very mild ones. Dogs without immunologic tolerance through continuous exposure can exhibit intense

pruritus associated with crusty papules over the whole body, but typically on the lower back, tail-head, posterior and inner thighs, but also flanks, neck and ears. The affected dogs will be restless and uncomfortable, constantly scratching, licking and rubbing, which can result in alopecia erythema, scaling and self-trauma and sometimes traumatic moist dermatitis (hot spots) [179].

Cats rather show a miliary dermatitis on face, neck and back, which is not directly due to flea bite, but manifestation of the allergic reaction, eczematous rash and potentially severe pruritus.

While normally, the diagnosis of flea infestation can be easily proved by presence of flea or flea excrements, in allergic animals often almost nothing can be found due to the extensive licking and self-grooming. IDT can support a presumptive claim of FAD, although the reliability of the result in the cat may be variable.

Treatment mostly consists of removing flea with insecticides such as fipronil, selamectin, pyrethroids etc. and additional anti-inflammatory and anti-pruritic medication upon necessity. Disinfection and cleaning of animal environment (sleeping place, blankets etc.) is recommended to eradicate the entire flea as well as reducing the exposure to flea bites to the absolute minimum [179]. Besides flea bite hypersensitivity, a hypersensitivity towards other insects is possible, e.g. to mosquitos, which shares most characteristics with FAD [165].

1.2.1.4. Food allergy/Cutaneous adverse food reaction (CAFR)

The pathogenesis of CAFR in cat and dog is not yet completely understood. It is also unclear how common this disease is. Furthermore, it should be differentiated between immune-modulated reactions due to food allergy and abnormal reaction without the immune system being involved due to food intolerance. In the former one, type I and type IV hypersensitivity reactions determine the categorization as food allergy. Non-seasonal pruritus is the main symptom for CAFR, which after exclusion of other allergic or parasitary diseases, has to be confirmed by a strict elimination diet trial [165]. The itching can be accompanied by gastrointestinal symptoms such as vomiting or diarrhea. By now, the most common offending food allergens are well-known: Dogs show the most adverse reactions against beef, dairy products, chicken and wheat. Cats are most sensitive toward beef, fish, chicken and dairy products [180]. The elimination diet starts with a complete change of nutrition, with either home cooked meals, hydrolyzed protein or

commercial protein. Important is, that the diet should preferably not contain any protein to which the cat or dog has been exposed before. For this purpose, horse or kangaroo or other relatively exotic protein sources can be used. The duration of the trial should be 6 to 8 weeks which gives enough time for complete remission in most cases. Afterwards the original diet will be gradually reintroduced. If symptoms reappear, the diagnosis for CAFR is possible [165, 180]. While in theory, this approach seems easy and promising; in practice the success depends to a great extent on pet owner compliance, strict abidance to the diet plan, suitability of the plan regarding the causative antigen and the presence or development of concurrent allergies[181].

1.2.1.5. Cutaneous leishmaniasis (dog)

Leishmania spp. are a genus of trypanosomes, obligate intracellular parasites, infectious to human and animals with *L. infantum* and *L. major* being the most common infective species. They are transferred via sand flies as vector, which occur in the Mediterranean and (sub)-tropical areas. The disease can get manifested as cutaneous, mucocutaneous and visceral leishmaniosis (CL, ML, VL). Dogs infected with the cutaneous form (Canine CL, CCL) act as a major parasite reservoir and contribute largely to human transmission of CL and VL in endemic regions. On the other hand, infected dogs are also a significant veterinary problem [169, 170].

After the infection through sand fly stings, the parasites multiply in macrophages and other cells of the MPS right at the infection site and spread from there. They can leave the skin and colonize organs like spleen, liver and bone marrow and cause chronic, recurrent and sometimes fatal symptoms [170, 182]. CL in dogs usually manifests as localized ulcerative, erosive lesions which either heal or become chronic and can lead to massive tissue destruction and necrotizing dermatitis and secondary infections by bacterial overgrowth [169, 170, 183, 184]. Genetic composition, especially T-cell and cytokine mediated immune response, has been shown to significantly influence the course and severity of the disease in the individual. Also variations in virulence of the parasite are considerable [170, 185].

The current therapy options for leishmaniosis in dogs are with pentavalent antimonials, allopurinol and amphotericin B limited and toxic, and more often

than not, the success is limited to clinical improvement but parasitological cure is not possible and relapse rates of up to 74% have been reported [185].

1.2.2. Horse

Horses are somewhat in between companion and livestock animal. While for many private owners their horse is more of a friend and leisure partner, for breeders and sport associations the horse is the main source of income and therefore has to be optimized to best functionality. Skin diseases can cause a heavy financial burden to them [172, 173] as they may lead to the horse not being able to be used (riding, driving, breeding, shows, circus...) anymore, and also needing extensive therapy. Skin diseases of the horse may be of bacterial or viral origin but also due to allergic and parasitic causes. One important example is summer eczema.

1.2.2.1. Insect bite hypersensitivity/Summer eczema

As for cats and dogs, also for horses, insect bite hypersensitivity is a very common skin associated disease. It is described in several countries with different names (summer eczema, sweet itch etc.) and in different horse breeds. It can strongly impair a horse's life quality, in some cases to such an extent, that the horse cannot be ridden anymore, and cause great financial loss to the owner [172, 173]. Although the problem is well known for different horse breeds and most horses can be affected to a certain degree, it seems that exported Icelandic horses are specifically susceptible compared to others, which can be explained by the fact, that in Iceland the causing insects do not occur and the horses are not familiar to those antigens [172, 173, 186]. Other affected horse races are the Finnhorse and New Forest Pony but also Arab horse and Thoroughbreds [173]. Summer eczema is an allergic reaction to the bite of *Culicoides* species, biting midges which are active during the grazing season. Horses affected by the disease suffer from severe pruritus (itching) especially around the mane, tail and hind regions which causes self-excoriation leading to open wounds and secondary infections. In severe cases, the lesions can be spread over the entire body. The tendency to develop the condition is not only breed associated but also transferred by parental inheriting. The symptoms usually start when the horse is around five years old, often correspondent to experiencing their third or fourth grazing season which can lead to gradually increasing sensitization. Another factor is the

frequency and amount of *Culicoides spp.* the horses are exposed to which strongly depends on country and area. Generally, low altitude, moisture and lee at pasture and open water in the neighborhood are connected to a higher prevalence and severity [172, 173, 186].

Treatment is challenging and mainly focuses on minimizing insect contact by keeping the horses inside during summer, insecticides and special blankets and relief of allergic symptoms with antihistamines and glucocorticoids.



Fig. 21: 16 year old warm blood gelding with severe summer eczema lesions
(Pictures friendly provided by Birgit Heichele)

1.2.3. Livestock

Livestock animals like swine, sheep, goats and cattle also suffer from skin associated diseases. Unlike in companion animals, the causative agents of these diseases are more often bacterial, viral or parasitic infections than allergic or hypersensitivity reactions. In many cases, poor hygienic conditions, bad husbandry and overcrowding, which result in distress, aggression and injuries, hence also in a weakened immune system, lead to epidemic disease outbreaks by opportunistic microorganisms [187, 188]. Ringworm, a zoonotic disease caused by dermatophyte fungi of *Trichophyton spp.* or *Microsporum spp.*, and mange [171] can serve to exemplify such an opportunistic infection. It should be also considered that with large numbers of animals in a relatively small space, naturally the transfer of diseases of every kind is facilitated [189, 190] and might happen very rapidly. Treatment in this condition is often difficult. One further example of a livestock skin disease with also considerable economic impact [174]

is exudative epidermitis.

1.2.3.1. Exudative epidermitis (greasy pig disease/facial dermatitis)

This disease, caused by *Staphylococcus hyicus*, affects usually neonatal piglets until 8 weeks [174], but also older pigs can be concerned, especially after mixing following weaning, and develop aggressive behavior and have to compete about resources, e.g. access to water. Infected pigs show brown and scaly lesions beginning on the head and neck area which can spread over the whole body and exude a greasy liquid. The moist patches acquire dust and soon look black in color. The disease can be differentiated in an acute and a chronic form. In the acute form, along with formation of lesions, the affected pigs rapidly exhibit depression and dehydration symptoms and might also die. Another rare form of this infection can cause generalized thickening and wrinkling of the skin so such an extent that euthanasia might have to be considered. Main causes for the outbreak of the disease are: Excessive fighting between piglets resulting in skin damage, high humidity in stables favoring the proliferation of the normally rather unproblematic *S. hyicus*, and generally poor hygiene conditions. A correlation with the prevalence of sarcoptic mange was described [171]. Treatment consists of systemic antibiotics and hydration with electrolyte solution in case of dehydration [171]. Death of piglets, decreased growth rates and reduced carcass value because of damaged hides due to lesions and scars, can cause significant financial loss to the owner [174].



Fig. 22: piglet with facial lesions. Greasiness and sticking dust is demonstrated.
(Picture friendly provided by Anna Müller-Keller)

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